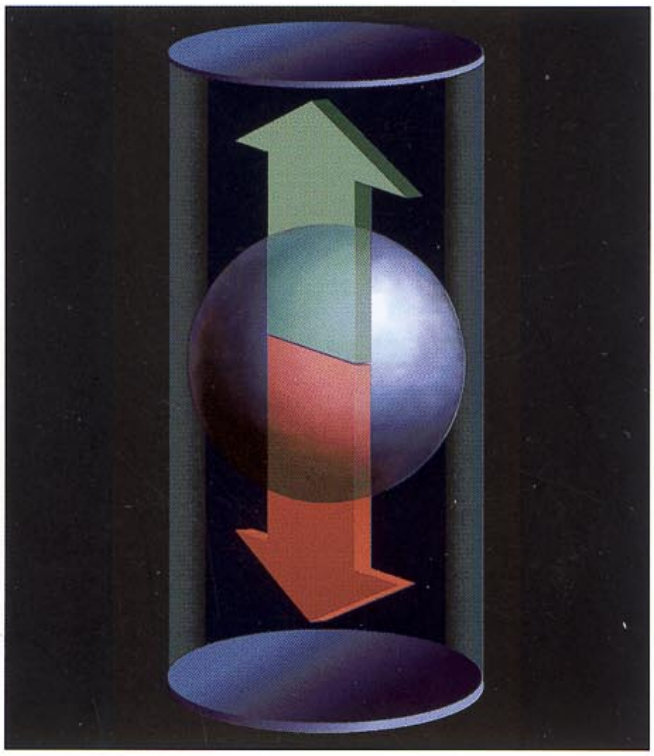
Expanded Bed Adsorption



Principles and Methods

Back to Collection

18-1124-26
Edition AB

amersham pharmacia biotech

1. Introduction to Expanded Bed Adsorption

This handbook introduces the principles of Expanded Bed Adsorption and serves as a practical guide to the use of STREAMLINE adsorbents and columns available from Pharmacia Biotech. Critical operating parameters will be discussed as well as principles for method design and optimization that will ensure maximum exploitation of this unique unit operation. The handbook is illustrated with examples of the different types of biological molecules which have been purified using Expanded Bed Adsorption.

The majority of biotechnology processes for producing pharmaceutical or diagnostic products involve the purification of proteins and peptides from a variety of sources. Those include bacteria, yeast and mammalian cell culture fluids, or extracts from naturally occurring tissue.

Typically, such purification schemes contain multiple unit operations, including a number of chromatographic steps to ensure the safe removal of critical impurities and contaminants. The type of product produced and its intended use will dictate the extent of purification needed. Each step in the recovery process will affect the overall process economy by increasing operational cost and process time, and also by causing loss in product yield. Careful selection and combination of suitable unit operations during the design phase may reduce the number of steps needed. To design-in the fewest possible processing steps thus offers the most efficient way of reaching high process economy in the overall production process.

The initial purification of the target molecule has traditionally been addressed by adsorption chromatography using a conventional packed bed of adsorbent. This necessitates clarification of the crude feed before application to the chromatography column.

The standard techniques used for removal of cells and/or cell debris have been centrifugation and microfiltration. The efficiency of a centrifugation step depends on particle size, density difference between the particles and the surrounding liquid, and viscosity of the feed-stock. When handling small cells, such as *E. coli,* or cell homogenates, small particle size and high viscosity reduce the feed capacity during centrifugation and sometimes make it difficult to obtain a completely particle-free liquid. To obtain a particle-free solution that can be further purified by traditional packed bed chromatography, centrifugation is usually combined with

microfiltration. However, microfiltration also has its drawbacks. Although microfiltration yields cell free solutions, the flux of liquid per unit membrane area is often dramatically decreased during the filtration process. Fouling of the microfiltration membranes is another problem that significantly adds to the operational cost.

The combined use of centrifugation and filtration often results in long process times or the use of comparatively large units causing significant capital expenditure and recurrent costs for equipment maintenance. It also results in significant product loss due to product degradation. Hence, direct adsorption from crude feed-stocks potentially offers significant reduction of time and cost compared to traditional processes.

An alternative to traditional clarification and packed bed chromatography is adsorption to a resin in a stirred tank. This technique can be used to advantage when recovering the target substance from a large volume of crude feed. In packed bed mode this would require a long sample application time and initial removal of particulate material to prevent clogging of the bed. This method has, for instance, been used for many years on a commercial scale for the isolation of plasma coagulation Factor IX with DEAE Sephadex (1). However, the well-mixed batch adsorption process is a single-stage adsorption procedure that requires more adsorbent to achieve the same degree of adsorption as in a multi-stage (multi-plate) process such as packed bed chromatography. Hence, a multi-plate process represents a more efficient use of the resin, which reduces the cost of the process.

Adsorption of the target molecule to an adsorbent in a fluidized bed also eliminates the need for particulate removal. Fluidized beds have been used in industry for many years for the recovery of antibiotics including batch-processing techniques for recovery of streptomycin (2) and semi-continuous systems for novobiocin (3). A method has also been published describing the successful capture of immunomycin from a *Streptomyce*s culture at large scale (4).

In the fully fluidized bed, channelling, turbulence and backmixing is extensive, constituting at the most a single equilibrium stage, i.e. showing characteristics very similar to a batch process in a stirred tank. The single equilibrium stage in a fluidized bed decreases the efficiency of the adsorption process with low recoveries (re-cycling needed), inefficient washing procedures and increased processing time.

Several attempts have been made to stabilize fluidized beds to accomplish a multi-stage fluidized bed reactor with separation characteristics similar to packed bed chromatography. The first approach used segmentation of the bed by insertion of a number of plates with suitably sized holes into the adsorption column (5). In another approach, magnetic adsorbent particles and a magnetic field over the fluidized bed column were used to stabilize the bed (6, 7). A substantial stabilization of the bed was achieved using magnetic adsorbents but the experiments were carried out at small laboratory scale and scaling up requires complicated and expensive equipment.

Draeger and Chase (8) were able to create a stable fluidized (expanded) bed, with chromatographic characteristics similar to a packed bed, by using conventional chromatographic adsorbents based on agarose in a column equipped with a purpose designed liquid distribution inlet giving a plug flow in the column. The application of mixtures of proteins and cells onto these expanded beds showed the potential of the technique for recovery of proteins from particle-containing feedstocks (9, 10, 11). The breakthrough capacity in such beds, expanded by a factor of two, was very similar to the breakthrough capacity in a packed bed. However, low flow velocities had to be applied to prevent the bed from expanding too much, which resulted in a low overall productivity. It was obvious from the experiments of Draeger and Chase that there was a need for particles with a higher sedimentation velocity to fully exploit the features of the expanded bed technology. In 1992, the first results from such prototype adsorbents, based on agarose, were reported (12, 13, 14, 15).

Commercially available adsorbents based on amorphous silica have also been investigated as possible candidates for use in expanded beds (16). These adsorbents are denser than agarose-based adsorbents, but the smaller bead size enables this material to expand to the same degree as beds of agarose beads at comparable flow velocities. A drawback of silica-containing material is the limited stability at high pH values, which makes it less suitable for biopharmaceutical production where high pH is frequently used for cleaning-in-place and sanitization-in-place procedures.

In 1993, Pharmacia Biotech introduced new types of chromatographic adsorbents and columns called STREAMLINE (17, 18), products specially designed for Expanded Bed Adsorption. STREAMLINE adsorbents and columns allow the formation of stable fluidized beds at high operating flow velocities. The first media introduced were two ion exchangers, STREAMLINE DEAE and STREAMLINE SP, both developed from the highly biocompatible agarose base matrix by the inclusion of an inert crystalline quartz core material to provide the required density. The defined particle size and density distribution of the STREAMLINE adsorbents, together with the specially designed STREAMLINE columns, yield expanded beds with well defined and consistent hydrodynamic properties, and with adsorption characteristics similar to those of packed beds of standard chromatography media (19, 20, 21, 22, 23).

The properties of Expanded Bed Adsorption make it the ultimate capture step for initial recovery of target proteins from crude feed-stock. The process steps of clarification, concentration and initial purification can be combined into one unit operation, providing increased process economy due to a decreased number of process steps, increased yield, shorter overall process time (24), reduced labour cost (25), and reduced running cost and capital expenditure (26).

Expanded Bed Adsorption has also proved to be a versatile tool that can be applied on all commonly used source materials. Successful processing by Expanded Bed Adsorption has been reported for *E. coli* homogenate (14, 24, 27-29, 40, 57, 58,

63, 74), *E. coli* lysate (30, 53, 57), E. coli inclusion bodies (31), secreted products from E. coli (32, 57), yeast cell homogenate (33, 34, 68), secreted products from yeast (41, 54, 56, 69, 75), whole hybridoma fermentation broth (35, 64, 70, 73), myeloma cell culture (65), whole mammalian cell culture broth (25, 36, 66, 67), milk (55, 60), animal tissue extracts (71), and culture supernatant from a continuous fluidized bed bioreactor (61).

Expanded Bed Adsorption by the use of STREAMLINE adsorbents and columns has also proven to be a scalable technique (37- 39, 66) that has found its way into the production halls of pharmaceutical manufacturers (40, 41).

A review of protein purification by adsorption chromatography in expanded beds has been published by Chase (42).

2. Principles of Expanded Bed Adsorption

Expanded bed adsorption is a single pass operation in which desired proteins are purified from crude, particulate containing feed-stock without the need for separate clarification, concentration and initial purification. The expansion of the adsorbent bed creates a distance between the adsorbent particals, i.e. increased voidage (void volume fraction) in the bed, which allows for unhindered passage of cells, cell debris and other particulates during application of crude feed to the column.

Basic principles of operation

The principle of expanded bed adsorption is shown in Fig. 1.

STREAMLINE adsorbent is expanded and equilibrated by applying an upward liquid flow to the column. A stable fluidized bed is formed when the adsorbent particles are suspended in equilibrium due to the balance between particle sedimentation velocity and upward liquid flow velocity. The column adaptor is positioned in the upper part of the column during this phase.

Crude, unclarified feed is applied to the expanded bed with the same upward flow as used during expansion and equilibration. Target proteins are bound to the adsorbent while cell debris, cells, particulates and contaminants pass through unhindered.

Weakly bound material, such as residual cells, cell debris and other type of particulate material, is washed out from the expanded bed using upward liquid flow.

When all weakly retained material has been washed out from the bed, the liquid flow is stopped and the adsorbent particles quickly settle in the column.

The column adaptor is then lowered to the surface of the sedimented bed. Flow is reversed and the captured proteins are eluted from the sedimented bed using suitable buffer conditions. The eluate contains the target protein, increased in concentration, clarified, partly purified, and ready for further purification by packed bed chromatography.

After elution, the bed is regenerated by washing it with downward flow in sedimented bed mode using buffers specific for the type of chromatographic principle applied. This regeneration removes the more strongly bound proteins which are not removed during the elution phase.

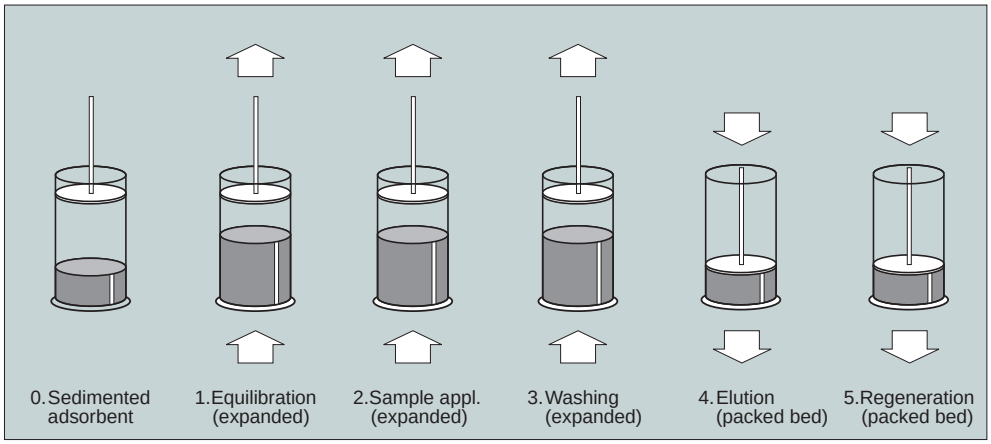


Fig. 1. Schematic presentation of the steps of expanded bed adsorption.

Finally a cleaning-in-place procedure is applied to remove non-specifically bound, precipitated, or denaturated substances from the bed, and restore it to its original performance. During this phase, a moderate upward flow is used with the column adaptor positioned at approximately twice the sedimented bed height.

Stable fluidization

Expanded bed adsorption is based on controlled stable fluidization, thus combining the hydrodynamic properties of a fluidized bed with the chromatographic properties of a packed bed. The fluidization allows particulate matter to pass through the bed unhindered. The expanded bed principle, i.e. the formation of stable fluidization with a minimum of back-mixing, channelling and turbulence in the bed, allows the formation of several mass transfer units or several theoretical plates in the expanded bed, mimicing the performance of a traditional packed chromatography column.

Results from studies of the hydrodynamic properties of expanded beds based on STREAMLINE media were reported by Johansson and Wnukowski (15). The investigation included three different sedimented bed heights, ranging from 56 mm to 236 mm, and flow velocities ranging from 100 to 300 cm/h generating a degree of expansion from 1.7 to 3 times the sedimented bed height. The general conclusion was that longitudinal mixing in the liquid phase was low and consistent, indicating stable fluidization and a plug flow liquid profile through the bed, i.e. a similar behaviour as in packed beds. The axial dispersion coefficients were in the range 1–6 x 10^{-6} m^2 s^{-1}. Discrepancies in the results were observed for the lowest flow velocity, which was explained by the bed not being purely random at low expansion

ratios. Instead, large conglomerates of particles are formed together with channels and paravoids, contributing to an unpredictable behaviour of the bed. It was also concluded that for the same flow velocity, the axial dispersion increased with the height of the bed, an effect that can be explained by the plug flow being retarded at the wall of the column creating radial variations of the bed voidages across the cross-section of the column.
Stability of expanded beds using STREAMLINE media was also verified by Wnukowski and Lindgren (13) by using a set up with a sedimented bed height of 225 mm, expanded 3.2 times at a linear flow velocity of 300 cm/h. They reported an axial dispersion coefficient of 9×10^{-6} m^2 s^{-1}.

Similarly, Batt et al (25) reported an average axial dispersion coefficient of 1.86×10^{-6} m^2 s^{-1} in a series of experiments on STREAMLINE SP using a flow velocity of 135 cm/h and a sedimented bed height of 8.6 cm.

Thömmes et al (64) reported results from hydrodynamic studies with a small scale column using a sedimented bed height of 6 cm. They concluded that a certain minimum flow velocity is required for development of a stable fluidized bed. Axial mixing was significantly lower at the highest flow velocity, compared with the lowest, corresponding to a decrease in Bodenstein number from 33 to 11 when the flow velocity was decreased from 375 cm/h to 200 cm/h in this specific experimental system. The axial dispersion coefficients were in the range $6–9 \times 10^{-6}$ m^2 s^{-1}.

Hence, the same order of magnitude of axial mixing has been reported by different investigators, demonstrating that stable fluidization can be achieved with STREAMLINE adsorbents. Sedimented bed height and linear flow velocity are critical process parameters that may have a significant impact on the performance of an expanded bed. The importance of sedimented bed height, flow velocity and other critical processing parameters, including the effect of physico-chemical properties of the crude feed stream, will be discussed in more detail in Section 3, Experimental Design and Section 4, Method Optimization.

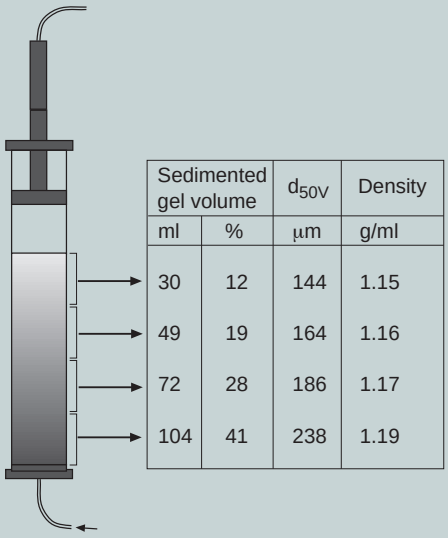
Design features

The adsorbent

Tailoring the chromatographic characteristics of an adsorbent for use in expanded bed adsorption includes careful control of the sedimentation velocity of the adsorbent beads. The sedimentation velocity is proportional to the density difference between the adsorbent and the surrounding fluid multiplied by the square of the adsorbent particle diameter. To achieve the high throughput required in industrial applications of adsorption chromatography, flow velocities must be high throughout the complete purification cycle. The first results reported from expanded bed adsorption using conventional chromatographic adsorbents based on agarose (8) revealed an obvious need for particles with higher sedimentation velocity to allow the operation of expanded beds at high flow velocities without the beads being carried out of the column by the lifting liquid flow.

STREAMLINE adsorbents are based on agarose, a material proven to work well for industrial scale chromatography. The macroporous structure of the highly cross-linked agarose matrices combines good binding capacities for large molecules, such as proteins, with high chemical and mechanical stability. High mechanical stability is an important property of a matrix to be used in expanded bed mode to reduce the effects of attrition when particles are moving freely in the expanded bed. The modified agarose matrix used in the manufacture of STREAMLINE adsorbents is less brittle than inorganic material such as some glass or ceramic materials. The mechanical stability of STREAMLINE adsorbents has been verified by repeated expansions and sedimentations and by subjecting the adsorbent to different types of shear force (43).

Particles made only of organic material have limited density and would need to have very large diameters for the high sedimentation velocity required. Such large particle diameters result in long diffusional path-lengths, which cause considerable mass transfer resistance, counteracting productivity. STREAMLINE adsorbents are therefore based on a composite particle containing an inert core material that is denser than organic materials. Such particles can be designed so that their sedimentation velocity is high also at a reasonable particle size. STREAMLINE adsorbents exhibit a Gaussian like distribution of particle size and particle density which is illustrated in Fig. 2. (work from Pharmacia Biotech (43)).



Sedimented gel volume		d_{50V}	Density
ml	%	μm	g/ml
30	12	144	1.15
49	19	164	1.16
72	28	186	1.17
104	41	238	1.19

Fig. 2. Distribution of beads of a STREAMLINE ion exchanger expanded 2.5 times with water at a flow velocity of 300 cm/h in a STREAMLINE 50 column (50 mm i.d.). (Work by Pharmacia Biotech.)

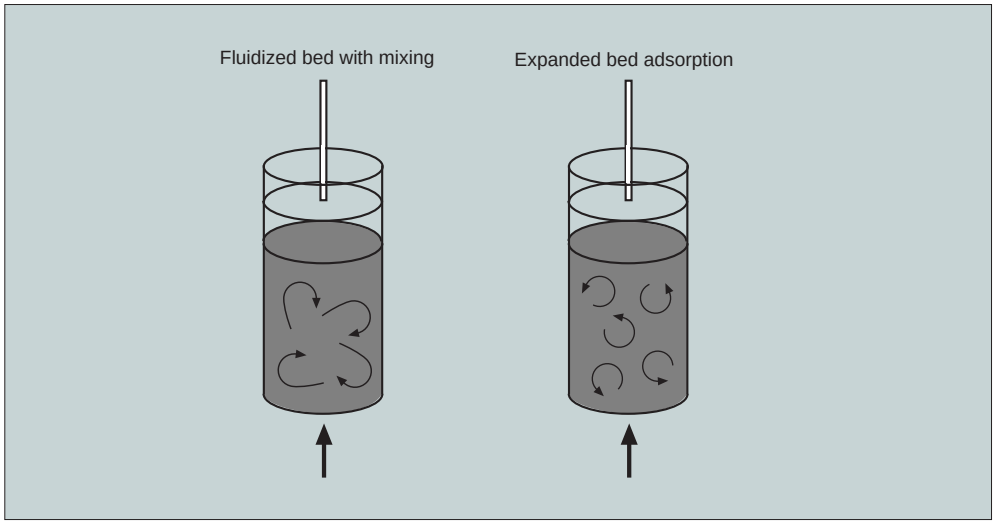


Fig. 3. Comparison of particle movement in a fluidized bed with extensive mixing with particle movement in an expanded bed. At the stable fluidization of an expanded bed, only small circular movements of the adsorbent beads can be seen.

This particle polydispersity is an important design factor contributing to the stability of the expanded bed. The size and density gradients position the beads at specific heights in the expanded bed depending on the sedimentation velocity of the individual adsorbent particles. The smaller, lighter particles move to positions at the top of the expanded bed, the larger, heavier particles to the bottom, resulting in a stable, uniform expansion. In other words, the beads find their ideal position in the column, which is the reason for the low axial dispersion in expanded bed adsorption as illustrated in Fig. 3.

The column

The column also has a significant impact on the formation of stable expanded beds. STREAMLINE columns are equipped with a specially designed liquid distribution system to allow the formation of a stable expanded bed. The need for a specially designed liquid distribution system for expanded beds derives from the low pressure drop over the expanded bed. Usually, the flow through a packed bed generates such a high pressure drop over the bed that it can assist the distributor in producing plug flow through the column. Since the pressure drop over an expanded bed is much smaller, the distributor in an expanded bed column must produce a plug flow itself. Consequently, it is necessary to build in an additional pressure drop into the distribution system. Besides generating a pressure drop, the distributor also has to direct the flow in a vertical direction only. Any flow in a radial direction inside the bed will cause turbulence that propagates through the column.

Shear stress associated with flow constrictions also requires consideration when designing the liquid distributor. Shear stress should be kept to a minimum to reduce the risk of molecular degradation.

Another function of the distribution system is to prevent the adsorbent from leaving the column. This is usually accomplished by a net mounted on that side of the distributor which is facing the adsorbent. The net must have a mesh size that allows particulate material to pass through and yet at the same time confine the adsorbent to the column.

The distributor must also have a sanitary design, which means that it should be free from stagnant zones where cells/cell debris can accumulate.

More information about STREAMLINE columns is found in Section 6, Product Guide.

Characteristics of expanded beds

Bed expansion

Fluidization occurs when particles are pushed upwards in a column at a velocity corresponding to their sedimentation velocity. The degree to which a bed expands, i.e. how far up in the column a particular bead is transported, is controlled by the size and the density of the adsorbent beads, the linear flow velocity of the mobile phase and the viscosity of the mobile phase.

The size and density of STREAMLINE adsorbents beads have been defined to allow optimal expansion at flow velocities that will ensure high productivity of the purification system. STREAMLINE adsorbents expand about 2 to 3 times in normal buffer solutions at room temperature at flow velocities ranging from 200 to 400 cm /h. These are considerably higher flow velocities than can be applied with unmodified agarose adsorbents to achieve the same degree of expansion, which is illustrated in Fig. 4.

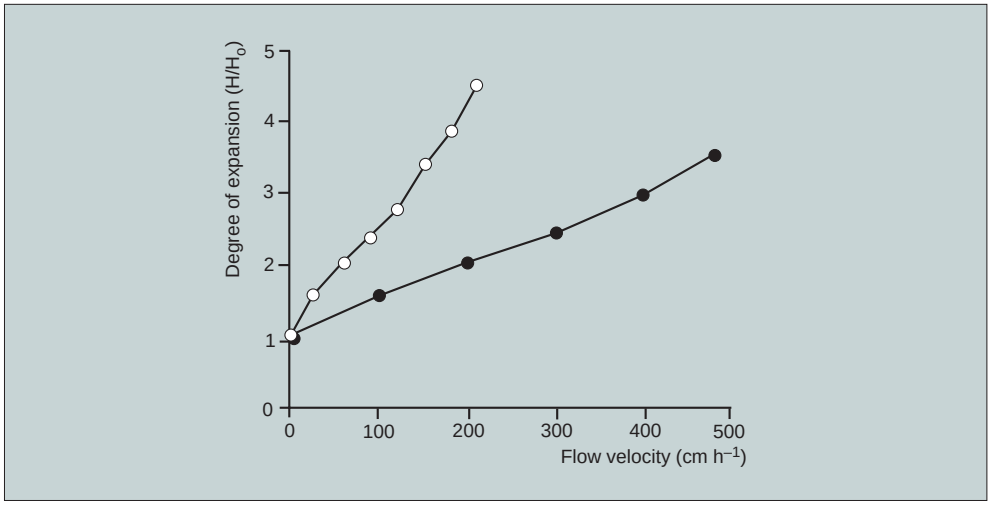


Fig. 4. Relative expansion at different flow velocities of STREAMLINE adsorbents (•) and an agarose matrix with the same particle size (100–300 µm) and agarose content (6%) but without inert core material (O). (Work by Pharmacia Biotech.)

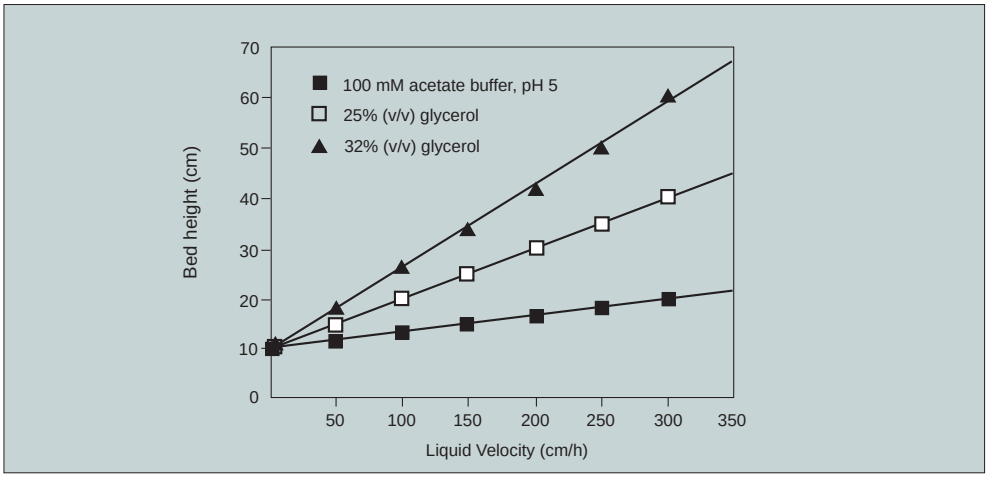


Fig. 5. Expanded bed height of STREAMLINE SP (sedimented bed height = 10 cm) with varying flow rate in a glycerol/buffer system in a STREAMLINE 50 column. (Reproduced with permission, from ref. 23.)

Note that absolute values for the degree of expansion will vary with working temperature and the buffer system used (liquid density and viscosity). The effect of increased viscosity of the buffer system is an increased degree of expansion. This is an important consideration during application of a crude and viscous feed material. The effect of increased viscosity of the mobile phase on bed expansion has been studied by Chang and Chase (23) and is illustrated in Fig. 5. The effect of viscosity and its implication in an expanded bed adsorption experiment will be discussed in more detail in Section 3, Experimental Design and Section 4, Method Optimization.

Adsorption characteristics

The stability of expanded beds based on STREAMLINE adsorbents provides adsorption characteristics similar to those of packed bed chromatography. As with packed bed chromatography, the available binding capacity depends on the molecular weight of the target substance, the binding strength to the ligands on the matrix, flow velocity and other conditions of the process. The absolute values for protein capacity given here are therefore only valid for specific proteins under defined flow velocities and process conditions.

Axial dispersion is usually an order of magnitude higher in an expanded bed compared with a packed bed. However, the adsorption characteristics of an expanded bed are very similar to a packed bed. This is demonstrated in Fig. 6 showing the breakthrough capacity for BSA on STREAMLINE DEAE adorbent in both expanded and packed bed mode. Only small differences are seen in breakthrough capacity and slope of the breakthrough curve. The same type of finding has been reported by Chase and Chang (20) using a similar test model with BSA and STREAMLINE DEAE adsorbent. They concluded that breakthrough curves in packed and expanded modes were indistinguishable indicating that adsorption performance is approximately the same in both.

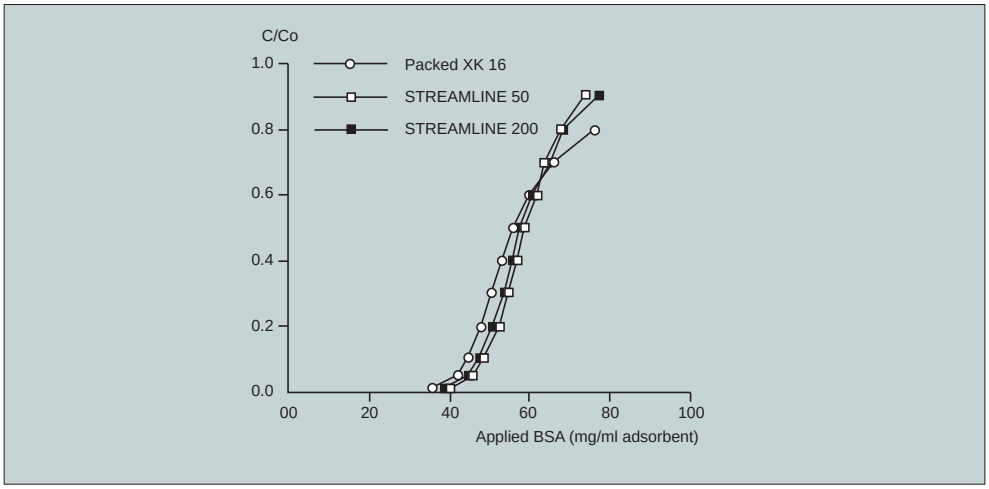


Fig. 6. Breakthrough curves for BSA on STREAMLINE DEAE in packed mode in an XK 16 column compared with expanded mode in a STREAMLINE 50 and STREAMLINE 200 column. (Work by Pharmacia Biotech.)

Fig. 7 compares the adsorption of lysozyme to STREAMLINE SP adsorbent in expanded mode with adsorption to SP Sepharose Fast Flow in packed mode using identical adsorption conditions. SP Sepharose Fast Flow is a cation exchange medium for packed bed chromatography that is frequently applied for the initial capture of proteins. A small difference in breakthrough capacity and steepness of the breakthrough curves can be observed. The avarage particle size of STREAMLINE SP adsorbent is 200 µm compared to 90 µm for the SP Sepharose Fast Flow adsorbent, which explains for the later breakthrough and steeper curve for SP Sepharose Fast Flow.

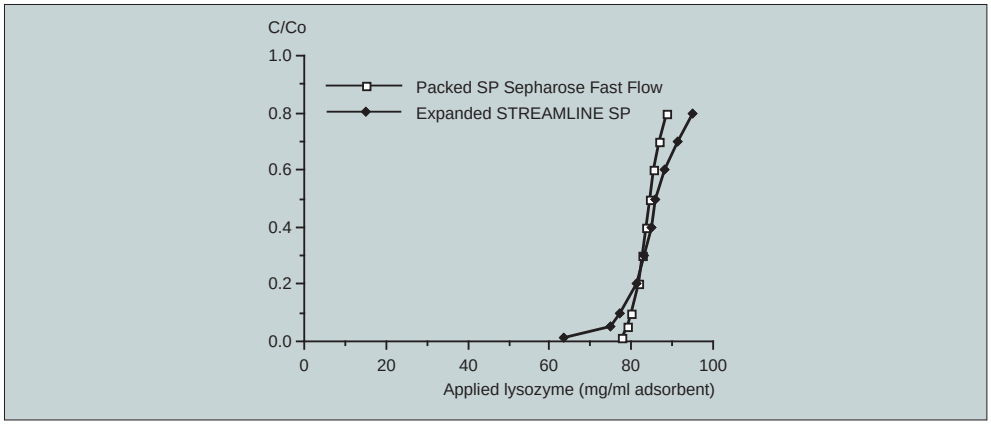


Fig. 7. Breakthrough curves for lysozyme on STREAMLINE SP and SP Sepharose Fast Flow at a flow velocity of 300 cm/h. (Work by Pharmacia Biotech.)

Operating pressure

Due to the high bed voidage (void volume fraction) of an expanded bed, typically around 0.7–0.8 (bed voidage of a packed bed is in the range 0.3–0.4), the restriction of flow is insignificant and consequently the operating pressures are extremely low during the whole operating cycle. Operating pressure is normally below 0.5 bar (50 kPa).

Evaluation of bed stability

Mastering the hydrodynamics of the expanded bed is critical for the performance of an expanded bed adsorption operation. The hydrodynamics of a stable expanded bed, run under well defined process conditions, are characterized by a high degree of reproducibility, which allows the use of simple and efficient test principles to verify the stability (i.e. functionality) of the expanded bed before the feed is applied to the column. The same type of test principles used to verify functionality of a packed chromatography column are used in expanded bed adsorption.

Visual inspection

When working with laboratory and pilot scale columns with glass tubes, visual inspection of movements in the expanded bed can be performed before feed application starts. The bed is stable when only small circulatory movements of the adsorbent beads are observed. Other movements may indicate turbulent flow or channelling, which leads to inefficient adsorption. Large circular movements of beads in the upper part of the bed usually indicate that the column is not in a vertical position. Channelling in the lower part of the bed usually indicates air under the distributor plate or a partially clogged distribution system. These visual patterns are illustrated in Fig. 8.

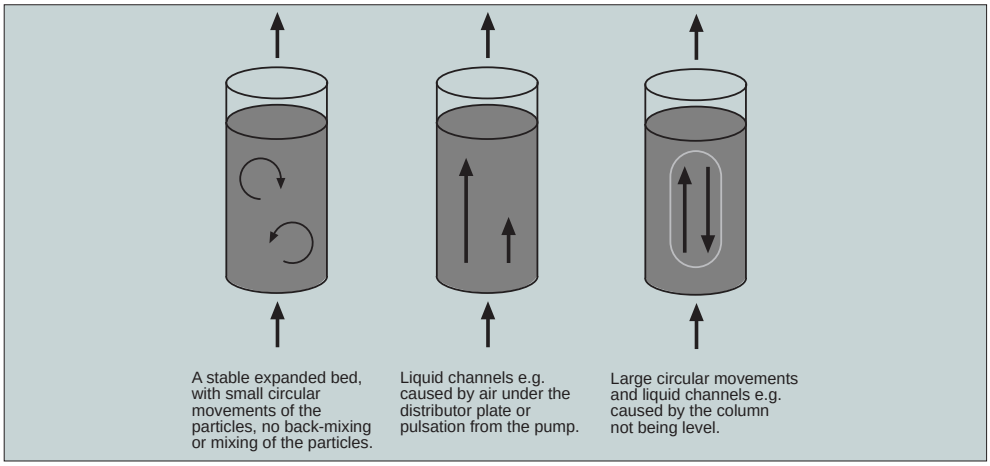

A stable expanded bed, with small circular movements of the particles, no back-mixing or mixing of the particles.
Liquid channels e.g. caused by air under the distributor plate or pulsation from the pump.
Large circular movements and liquid channels e.g. caused by the column not being level.

Fig. 8. Visual patterns of movement of adsorbent beads in an expanded bed.

Visual inspection alone, however, does not give the complete picture of the flow distribution across the entire column cross-section. Bed stability should be evaluated by more accurate techniques, such as measuring the degree of expansion and number of theoretical plates, before each run.

Measuring the degree of expansion

Measuring the degree of expansion is a quick and useful measure of bed stability, although less accurate than determining the number of theoretical plates. The degree of expansion is determined from the ratio of expanded bed height to sedimented bed height, H/H0, as defined in Fig. 9. If the degree of expansion differs from the expected value, it may indicate an unstable bed. Absolute values for the degree of expansion can only be compared if the buffer system (liquid density and viscosity) and temperature are constant between runs. A significant decrease in the degree of expansion may indicate poor stability or channelling due to trapped air under the distributor plate, infection or fouling of the adsorbent, the column not being in a vertical position, or a blocked distributor plate.

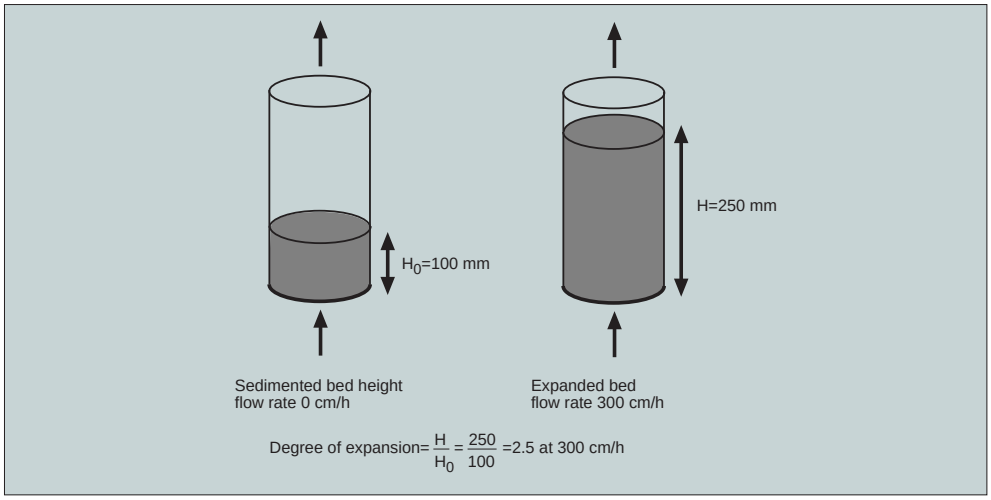


Fig. 9. Definition of the degree of expansion.

Number of theoretical plates

The Residence Time Distribution (RTD) test is a tracer stimulus method that can be used to assess the degree of longitudinal axial mixing (dispersion) in the expanded bed by defining the number of theoretical plates. A dilute acetone solution is used as a tracer input into the fluid entering the column. The UV absorbance of the acetone is measured in the exit stream from the column. The number of theoretical plates are calculated from the mean residence time of the tracer in the column and the variance of the tracer output signal, representing the standard band broadening of a sample zone. The RTD test is a simple but efficient tool for function testing

complete systems. If used to test systems before feed application, the risk of wasting valuable feed is reduced considerably. The test should be performed with the buffer and flow rate that are to be used during process operation. Note that when using a small tracer molecule (such as acetone) with a porous adsorbent (such as STREAMLINE media) the measurement of RTD is a function of tracer permeation in the matrix pores in addition to the actual dispersion in the liquid phase.

A description of the test procedure and calculations used to determine the number of theoretical plates when performing the test on a negative step input signal is given below.

1) When the bed is fully expanded at the test flow rate, mark the expanded bed height on the column and continue pumping buffer.
2) Lower the adaptor so there is about 0.5 to 1 cm between the net and the expanded bed surface.
3) Start the recorder and UV monitor. When the baseline is stable, change to buffer-acetone mixture (0.25% v/v) and wait for the positive step input UV-signal (Fig. 10).
4) Change back to buffer when the UV signal is stable at maximum absorbance (100%). Mark this change on the recorder paper ("*mark*" in Fig. 10)
5) Wait for the negative step input UV-signal and allow the signal to stabilize at the baseline level (0%).
6) Calculate the number of theoretical plates (N) from the negative input UV-signal.

$$N = t^2/\sigma^2$$

where

t = mean residence time

σ = standard deviation

t is the distance from the mark in step 4 of the test procedure to 50% of the maximum UV-signal (Fig. 10).

σ is measured as half the distance between the points 15.85% and 84.15% of the maximum UV-signal (Fig. 10).

A difference of more than ±20% in the number of theoretical plates between two runs performed under identical test conditions indicates that the bed is not stable.

A reasonably good value for N is within the range 25–30 at a sedimented bed height of 15 cm, using a nominal flow velocity of 300 cm/h. This corresponds to a plate number of 170–200 N/m.

If the mean residence time as calculated above is significantly shorter than the theoretical residence time (hydrodynamic residence time calculated from the reactor volume and the applied flow rate), it indicates insufficient fluidization and the formation of flow channels in the lower part of the bed, causing early breakthrough of the buffer front.

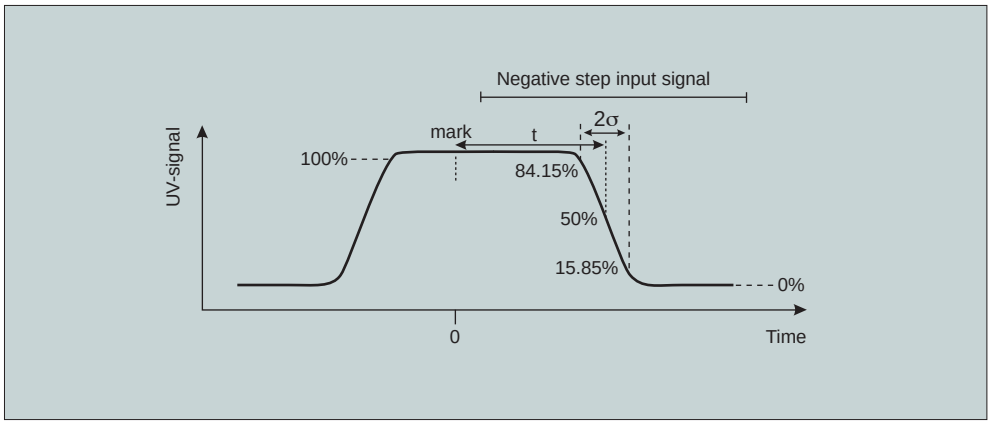


Fig. 10. UV-signal recording during the test procedure for the determination of the number of theoretical plates.

Note: Large dead volumes in a complete configuration with column, pumps, valves and tubing may cause low values for the number of theoretical plates.

The positive step signal (from 0% to 100%) is not recommended for evaluation as the reproducibility of the results is not as high as for the negative step signal.

Measurement of the theoretical plate number can also be done by injecting a volume of the tracer as a pulse.

3. Experimental Design

A complete downstream processing scheme will consist of different stages (Fig. 11). Each stage serves a specific purpose related to the state of the feed material, the estimated final scale of operation, processing time requirements, and the amount of purification needed at that stage. Each stage will be represented by one or several unit operations and may be referred to as Capture, Intermediate Purification and Polishing.

Capture is the first critical stage in all bioproduct recovery processes. It may include a number of different unit operations such as cell harvesting, product release, feedstock clarification, concentration and initial purification. For highest possible productivity and process economy of the overall process, the ultimate goal will be to reach the targets for purity and yield in as few steps as possible and with the simplest possible design.

Expanded bed adsorption technology with STREAMLINE is specifically designed to address the problems related to the beginning of the downstream sequence and may serve as the ultimate Capture step since it combines clarification, concentration and initial purification into one single operation.

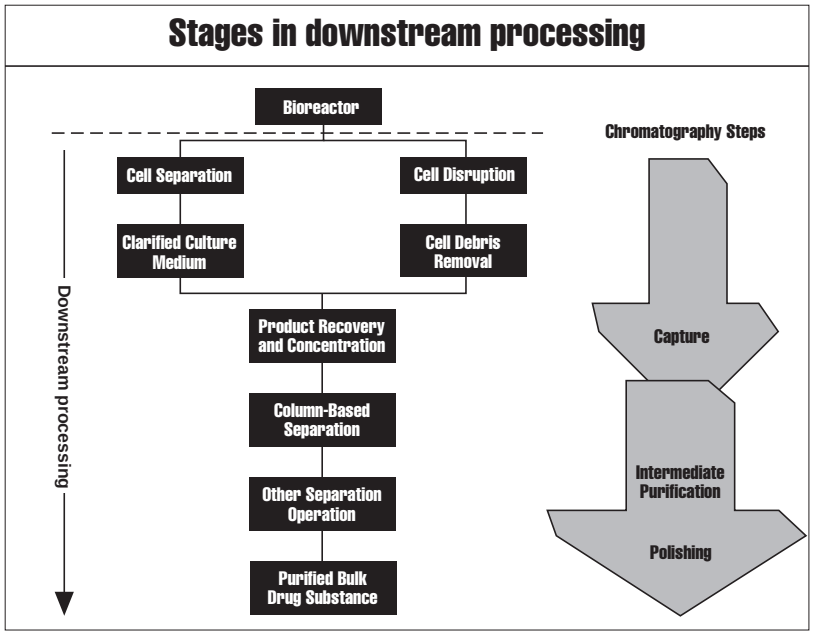


Fig. 11. Schematic diagram of a bioproduct recovery process showing the different stages in downstream processing.

Strategic considerations

The overall purpose of the Capture stage is to rapidly isolate the target molecule from critical contaminants, remove particulate matter, concentrate and transfer to an environment which conserves potency/activity. Some typical Capture objectives are:

- Stabilize the product
- Remove proteases, glycosidases, etc.
- Remove solids
- Remove cells
- Remove water
- Remove bulk quantities of proteins, nucleic acids and carbohydrates
- Prepare for (further) chromatography

At the Capture stage, high throughput (i.e. capacity and speed) is very important for processing large sample volumes, keeping the scale of equipment as small as possible and giving the shortest possible cycle time.

Processing time is critical at this stage since fermentation broths and crude cell homogenates contain proteases and glycosidases that reduce product recovery and produce degradation products that may be difficult to remove later. To prevent product degradation, it is desirable to minimize the time the product is exposed to such enzyme activity. Adsorption of the target molecule on a solid adsorbent decreases the likelihood of interaction between degradative enzymes and susceptible intramolecular bonds in the target molecule. For this reason, it is desirable to bind the target molecule as quickly as possible to an adsorbent. This can be achieved with STREAMLINE expanded bed adsorption, as crude feed can be applied directly to the adsorbent without prior clarification or concentration.

Expanded bed adsorption also increases productivity at the Capture stage due to the reduced overall processing time and increased yields that result from reducing the number of unit operations applied.

Furthermore, process economy benefits from the reduction of costs associated with labour, consumables, maintenance and capital expenditure when comparing a single expanded bed adsorption step with a multiple-step approach based on the traditional sequence of centrifugation, filtration, and packed bed chromatography.

Critical parameters

The critical parameters in expanded bed adsorption can be divided into chemical parameters and physical parameters.

Chemical parameters are the parameters related to the selectivity and capacity of the separation process and include pH, ionic strength, types of ions and buffers used. The influence on separation performance of these parameters is virtually the same in expanded bed adsorption as in traditional packed bed chromatography.

Physical parameters are the parameters related to the hydrodynamics and stability of a homogeneous fluidization in the expanded bed. Some physical parameters are related to the broth composition, e.g. cell density, biomass content and viscosity. Others are related to operating conditions such as temperature, flow velocity and bed height.

Chemical parameters are optimized during method scouting in packed bed mode as described under “Experimental strategy”. Some of the chemical parameters, such as pH and conductivity, are worth investigating thoroughly to optimize interfacing fermentation and expanded bed adsorption.

For example, high conductivity feed-stock applied directly to an ion exchange adsorbent would reduce capacity. This situation may call for dilution before application to the expanded bed to achieve maximum loading capacity.If conductivity is minimized at the end of the fermentation step, dilution is unnecessary. This results in less feed volume and shorter feed application time. In an intracellular system, conductivity of feed-stock can be reduced by running the homogenization step in water or a dilute buffer.

The pH range defined during method scouting should also be verified in expanded bed mode since reduced pH in some systems may cause aggregation of biomass. This aggregation can block the column distribution system causing poor flow distribution and an unstable bed.

Physical parameters are optimized in expanded bed mode since they relate to the hydrodynamic properties of the expanded bed.

Cell density and biomass content both affect viscosity, which may reduce the maximum operational flow rate by over-expanding the bed.

Temperature also affects the viscosity and, hence, the operational flow rate in the system. Fig. 12 shows the effect of temperature on the degree of expansion in a buffer system. Increased temperature can improve binding kinetics as demonstrated in Fig. 13, which shows breakthrough curves for BSA at two different temperatures. Optimization experiments are usually carried out at room temperature but a broth taken directly from the fermentor may have a higher temperature. This difference in temperature must be considered when basing decisions on results from small scale experiments. It may be worth testing feed application at elevated temperatures since reduced viscosity and improved binding kinetics can allow a higher flow rate and thus shorter cycle times.

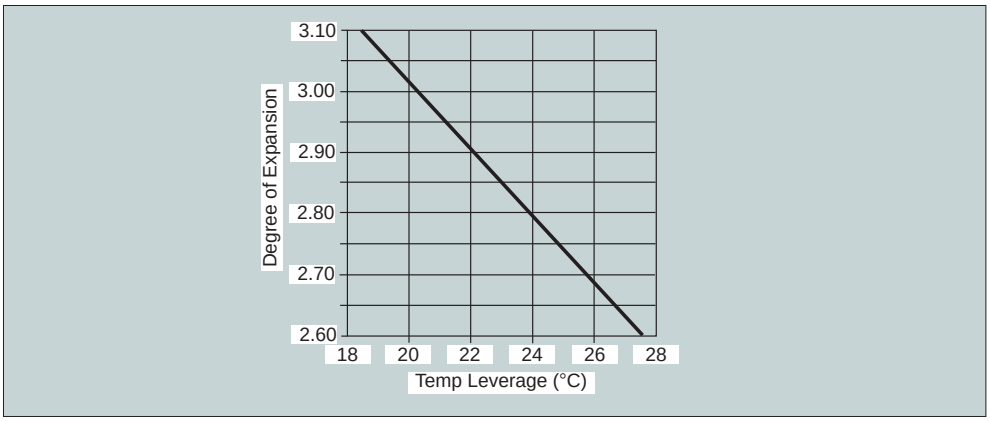


Fig. 12. Degree of expansion with varying temperature.

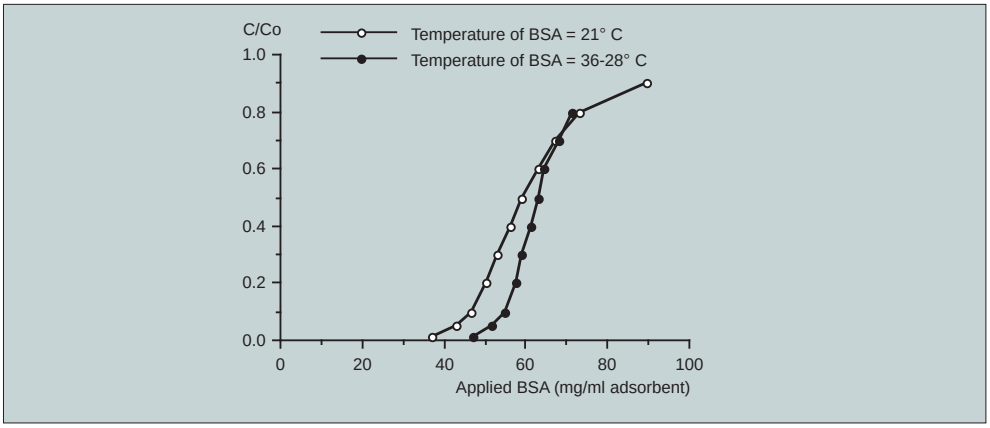


Fig. 13. Breakthrough curves for BSA on STREAMLINE DEAE at different temperatures (Work by Pharmacia Biotech.)

The effect of critical parameters on the different stages of an expanded bed adsorption step is discussed in more detail under "Feed characteristics" and in Section 4, Method Optimization.

Feed characteristics

The most critical aspect of the design of an expanded bed adsorption process concerns interfacing of process conditions with the properties of the starting material. This is important in any type of downstream process, but is of particular importance in expanded bed adsorption since the interaction of the raw feed with an adsorbent is so much more complex than the traditional application of clarified, pretreated feed to a packed bed of adsorbent. Different starting materials affect both the chromatographic and hydrodynamic performance of an expanded bed in completely different ways. The content of a feed-stock depends on the source material and its handling. In the case of a recombinant system, it also depends on the location of the accumulated product in the producing organism. A good understanding of the characteristics of different source materials and the results of their handling/processing is helpful to process development and optimization.

Table 1 lists some of the common characteristics of recombinant feed-stocks used to produce pharmaceuticals and other bioproducts.

A significant effect on the performance of expanded bed adsorption is whether the target molecule is secreted from the production organism into the culture medium or if it is accumulated intracellularly in the producing organism.

Secretion systems generate dilute, low viscosity feed-stock that contains rather low amounts of protein and intracellular contaminants, thus providing favourable conditions for downstream processing.

Intracellular systems, on the other hand, generate feed-stocks rich in intracellular contaminants and cell wall/cell membrane constituants. Along with the nutrient broth, these contaminants pose a greater challenge during the optimization phase of expanded bed adsorption. Much of the nutrient broth and associated contamination can be removed prior to cell lysis by thorough washing of the cells, but such steps introduce additional costs to the process.

The main source of contaminants in feed where the target molecule is located within the host cell is the complex cell membrane that has to be disrupted to release the target molecule.

Bacterial and yeast cell walls have a high polysaccharide content that can nucleate into larger structures that foul solid surfaces. Proteins and phospholipids are other integral parts of such cell walls that will be released upon cell disintegration. Bacterial cell walls are particularly rich in phospholipids, lipopolysaccharides, peptidoglycans, lipoproteins and other types of large molecules that are associated with the outer membrane of a bacterial cell. These contaminants may complicate downstream processing by fouling the chromatographic adsorbent. This type of

Table 1. Characteristics of feed-stocks according to the location of the product in the recombinant organism.

E. coli	**Yeast**	**Mammalian cells**
Secreted - Dilute, low viscosity feed containing low amounts of protein. Proteases, bacterial cells and endotoxins are present. Cell lysis often occurs with handling and at low pH. DNA can be released and cause high viscosity.	*Secreted* - Dilute, low viscosity feed containing low amounts of protein. Proteases and yeast cells are present.	*Secreted* - Dilute, low viscosity feed containing low amounts of protein. Proteases and mammalian cells are present. Cell lysis often occurs with handling and at low pH. DNA can be released and cause high viscosity. Cell lysis can also release significant amounts of lipids. Agglomeration of cells can occur.
Cytoplasmic - Cell debris, high content of protein. Lipid, DNA and proteases are present. Very thick feedstock which needs dilution. Intact bacterial cells and endotoxins are present.	*Cytoplasmic* - Cell debris, high content of protein. Lipid, DNA and proteases are present. Very thick feedstock which needs dilution. Intact yeast cells are present.	*Cytoplasmic* - Unusual location for product accumulation.
Periplasmic - Cell debris, high content of protein. Lipid and proteases are present. Thick feedstock which needs dilution. DNA is present if cytoplasmic membrane is pierced. Intact bacterial cells and endotoxin are present.	*Periplasmic* - Not applicable to yeast cells.	*Periplasmic* - Not applicable to mammalian cells.
Inclusion body - Cell debris, high content of protein. Lipid and proteases are present. Very diluted solutions after renaturation. Intact bacterial cells, DNA and endotoxin are present. Precipitation of misfolded variants occurs in a time dependent manner.	*Inclusion body* - Not applicable to yeast cells.	*Inclusion body* - Not applicable to mammalian cells.

contaminant may also be present as charged particulates that can act as ion exchangers and adsorb proteins, especially basic ones, if the ionic strength of the homogenate is low. This problem is, however, not specifically related to expanded bed adsorption and should be addressed when selecting conditions for cell disruption.

The main concern when processing a feed based on a secretion system would be to maintain intact cells, thereby avoiding the release of cell membrane components and intracellular contaminants such as DNA, lipids and intracellular proteins that may

foul the adsorbent or block the inlet distribution system of the column. Release of intracellular proteases is a further concern since it will have a negative impact on the recovery of biologically active material.

Animal cells lack a cell wall, which makes them more sensitive to shearing forces than microbial cells. The mammalian cell membrane is composed mainly of proteins and lipids. It is particularly rich in lipids, composing a central layer covered by protein layers and a thin mucopolysaccharide layer on the outside surface. Due to the high membrane content of mammalian cells, lysis can complicate the downstream process by causing extensive lipid fouling of the adsorbent. Another consequence of cell lysis is the release of large fragments of nucleic acids, which can cause a significant increase in the viscosity of the feedstock or disturb the flow due to clogging the column inlet distribution system. Nucleic acids may also bind to cells and adsorbent causing aggregation in the expanded bed. These types of contamination also lead to problems in traditional processing where they cause severe fouling during microfiltration.

Hybridoma cells are generally considered to be particularly sensitive to shear forces resulting from vigorous agitation or sparging. In contrast, CHO cells have relatively high resistance to shear rates and a good tolerance to changes in osmotic pressure.

The use of expanded bed adsorption reduces the amount of cell lysis that occurs, as compared with traditional centrifugation and cross-flow filtration unit operations, since the cells are maintained in a freely-flowing, low shear environment during the entire capture step. Nevertheless, it is important to actively prevent cell lysis during processing, for instance by avoiding exposure to osmotic pressure shocks during dilution of the feed-stock and by minimizing the sample application time.

Non-secreted products sometimes accumulate intracellularly as inclusion bodies, which are precipitated protein aggregates that result from over-expression of heterologous genes. Inclusion bodies are generally insoluble and recovery of the biologically active protein requires denaturation by exposure to high concentration of chaotropic salts such as guanidine hydrochloride or dissociants such as urea. The subsequent renaturation by dilution provides very large feedstock volumes. Expanded bed adsorption can be advantageous since precipitation of misfolded variants increases with time, which usually causes problems for traditional packed bed chromatography. Even after extensive centrifugation of the feed-stock, precipitation continues and may finally block a packed chromatography bed.

When a non-secreted product accumulates in the periplasmic compartment, it can be released by disrupting the outer membrane without disturbing the cytoplasmic membrane. Accumulation in the periplasmic space can thus reduce both the total volume of liquid to be processed and the amount of contamination from intracellular components. However, it is usually very difficult to release the product from the periplasmic space without piercing the cytoplasmic membrane and thereby releasing intracellular contaminants such as large fragments of nucleic acids, which may significantly increase the viscosity of the feed-stock.

In traditional downstream processing, the initial unit operations often include some type of pre-treatment to remove contaminants such as lipids and DNA to reduce fouling the adsorbent and increase the working life of the column. In expanded bed adsorption, these problems are addressed by careful selection of an efficient cleaning-in-place protocol to apply after each purification cycle. Cleaning-in-place procedures and other measures to reduce the effect of cells, cell debris and contaminants on the chromatographic and hydrodynamic properties of the expanded bed, will be further discussed in Section 4, Method Optimization.

Experimental strategy

The different phases of developing an optimized STREAMLINE expanded bed adsorption unit operation are listed in Table 2.

Table 2. The different phases of developing an expanded bed adsorption unit operation.

	Method scouting	**Method optimization**	**Process verification**	**Production**
Purpose	Screening of binding and elution conditions using clarified material in packed bed mode	Optimization of binding, elution, wash and CIP, using unclarified material in expanded mode at small scale	Verification at pilot scale Production for clinical trials	Production at full scale
Column	XK 16 or XK 26	STREAMLINE 25	STREAMLINE 50 STREAMLINE 200	STREAMLINE CD (custom designed)
Sedimented bed volume (litres)	0.02–0.15	0.05–0.15	0.2–9	up to several hundred litres

Method scouting

Method scouting, i.e. defining the most suitable STREAMLINE adsorbent and the optimal conditions for binding and elution, is performed at small scale using clarified feed in packed bed mode. Selection of adsorbent is based on the same principles as in packed bed chromatography. The medium showing strongest binding to the target protein while binding as few as possible of the contaminating proteins, i.e. the medium with the highest selectivity and/or capacity for the protein of interest, will be the medium of choice. Regardless of the binding selectivity for the target protein, STREAMLINE adsorbents are compatible with any type of feed material. However, when purifying proteins from mammalian cell culture systems by ion exchange chromatography, STREAMLINE SP is recommened as the first

choice. This is because of the high density of negatively charged glycoproteins on the surface of mammalian cells. These may interact with the positively charged surface of an anion exchanger such as STREAMLINE DEAE. The effect of this will be more severe above pH 7.

Suitable columns are XK 16 or XK 26 columns providing sedimented bed volumes of up to 0.15 litres (XK 26 at a maximum sedimented bed height of 30 cm). Nominal bed height is 15 cm which gives a sedimented bed volume of 0.03 litres in an XK 16 column and 0.08 litres in an XK 26 column.

The flow velocity during method scouting should be similar to the flow velocity to be used during the subsequent experiments in expanded mode. The nominal flow velocity for STREAMLINE expanded bed adsorption is 300 cm/h. This may need adjustment during optimization, depending on the properties of the feed-stock.

A small amount of clarified feedstock is applied to the packed bed at various binding conditions to define those that provide the optimal selectivity and capacity for the target protein. Elution can be performed step-wise or by applying a gradient. Linear gradients are applied in the initial experiments to reveal the relative binding of the target molecule versus the contaminants. This information can be used to optimize selectivity for the target molecule, i.e. to avoid binding less strongly bound contaminants. It can also be used to define the step-wise elution to be used in the final expanded bed.

When selectivity has been optimized, the maximum dynamic binding capacity is determined by performing breakthrough capacity tests using the previously determined binding conditions. The breakthrough capacity determined at this stage will give a good indication of the breakthrough capacity in the final process in the expanded bed, as has been discussed previously in Section 2.

Method optimization

Method optimization for the expanded mode is performed on small scale using crude, unclarified feed. A suitable column is the STREAMLINE 25 (25 mm i.d.), which provides a sedimented bed volume of up to 0.15 litres at a maximum sedimented bed height of 30 cm. A nominal bed height of 15 cm gives a sedimented bed volume of 0.074 litres in a STREAMLINE 25 column.

The purpose of the method optimization in expanded mode is to examine the effects of the crude feed on the stability of the expanded bed and on the chromatographic performance. If necessary, adjustments are made to achieve stable bed expansion with the highest possible recovery, purity and throughput.

During method optimization, the process should be challenged by applying a sample load close to the maximum sample load as defined in the breakthrough study performed at the method scouting phase. Challenging the process gives an

identification of critical process parameters and reveals what cleaning procedures are necessary to restore bed performance between runs. Proper cleaning ensures repeated use of the adsorbent over a large number of purification cycles.

When the effects of the crude feed on the expanded bed have been examined, the appropriate adjustments made, and the flow rate set for feed application, optimal loading of crude feed in expanded bed mode should be determined by performing a breakthrough capacity study. This is similar to the breakthrough capacity studies for clarified feed previously performed in packed bed mode.

The various problems that may be encountered during the method optimization phase are discussed in more detail in Section 4, Method Optimization.

Process verification

Verification of the optimized method for expanded bed mode can be carried out by scaling up to pilot scale using STREAMLINE 50 (50 mm i.d.) or STREAMLINE 200 (200 mm i.d.) columns, providing sedimented bed volumes of up to 9 litres (STREAMLINE 200 at a maximum sedimented bed height of 30 cm). A nominal bed height of 15 cm gives a sedimented bed volume of 0.29 litre in a STREAMLINE 50 column, and 4.7 litres in a STREAMLINE 200 column.

The principle for scale up is similar to that used in packed bed chromatography. Scale up is performed by increasing the column diameter and maintaining the sedimented bed height, flow velocity and expanded bed height. This preserves both the hydrodynamic and chromatographic properties of the system.

Scalability and scale up

In any type of chromatographic process, a successful scale up to full production can only be achieved by designing in scalability during the method development stage. Designing in scalability has to do with the careful selection of suitable media, buffers, chemicals, columns and system hardware, and with building robustness into the process at the early stages of method optimization. To assure scalability and robustness, sources of variation have to be defined, characterized, tested and, ideally, eliminated. Sources of variation that cannot be eliminated must be carefully controlled by setting specifications for the upper and lower limits of all critical process parameters. Safety margins have to be built into the process control parameters, based on challenge tests performed at the upper and lower limits of normal variations. The most critical sources of variation in expanded bed adsorption are related to the feed material, e.g. variations in product concentration, concentration of contaminating proteins, biomass content, viscosity, conductivity, cell lysis, nucleic acids, etc. Other sources of variation are related to the process

conditions, e.g. raw materials, buffer preparation, equipment, personnel, etc. Time may also be a critical source of variation since holding times between different steps, feed application time, etc., can vary, especially with scale up.

When scalability is considered from the start and built into the process during the method development work, actual scaling up is usually a straightforward process. The strategy is to maintain all the parameters that are related to the chromatographic and hydrodynamic performance, such as sedimented bed height, expanded bed height, flow velocity, sample load/volume of adsorbent, and volume of process buffers expressed in terms of the number of sedimented adsorbent bed volumes.

Some system factors may affect performance after scale up and may call for fine tuning of the process, such as adjustment of equilibration volume, wash volume, and elution volume.

A major concern when scaling up an expanded bed adsorption step is the column, especially the inlet and outlet liquid distribution systems. The most critical design parameters are the number of inlets and the extent of the pressure drop generated by the distribution system. A certain pressure drop has to be built into the distribution system for formation of plug flow. A large industrial column requires a higher pressure drop and a greater number of inlets than a small laboratory scale column. These two parameters have to be adjusted with the dimension of the column. Other important design parameters are the chemical resistance of the wetted material and the hygienic design. High chemical resistance allows the use of harsh chemicals during cleaning-in-place procedures. A hygienic design eliminates stagnant zones in the column where cells and cell debris can be trapped.

Scale up to final production is performed in STREAMLINE CD (custom designed) columns. These columns are designed with a distribution system that ensures the same distribution of flow, and the same stability of the expanded bed, as the laboratory scale and pilot scale columns used during method optimization. Consistent hydrodynamic and chromatographic performance has been verified in columns with inner diameters up to 1200 mm, providing sedimented bed volumes of more than 150 litres at a sedimented bed height of 15 cm.

Processing data from such a verification study (39) are shown in Table 3. The feed material used in this study was based on BSA spiked into a suspension of baker's yeast. The concentration of yeast in the feed was 4-6% dry weight. Expansion, equilibration, sample application and wash were performed at a flow velocity of 300 cm/h using 20 mM Tris, pH 7.5. Elution was performed by a single step procedure using a solution of 1.0 M NaCl in equilibration buffer. Elution was performed in packed bed mode, using downward flow, at a flow velocity of 100 cm/h. The adsorbent used was STREAMLINE DEAE and the amount of adsorbent used at each scale corresponded to a sedimented bed height of 15 cm. The method

development work was performed at laboratory scale on a STREAMLINE 25 column (25 mm i.d.). The established process was then verified in a pilot scale set-up using a STREAMLINE 200 column (200 mm i.d.), before scaling up to production on a STREAMLINE CD column with an internal diameter of 600 mm, followed by a second scale up step on a STREAMLINE CD with an internal diameter of 1200 mm. Scalability was verified by consistency in yield as shown in Table 3, and by consistency of chromatographic performance as judged by the appearance of chromatographic curves and analytical gel filtration of collected product peaks. RTD testing and breakthrough capacity determination gave further evidence of consistent performance at different scales, see Fig. 14. The discrepancy in wash volume between the two runs on the STREAMLINE 600 column is due to the fact that the adaptor was lowered to the bed surface earlier during the wash phase in run 2 compared with run 1. This change resulted in a significant reduction in consumption of wash buffer.

Table 3. Summary of process data from a scale-up verification study.

Scale	Sample volume (L)	Dry weight (%)	Load (g BSA/litre adsorbent)	Wash volume (SBV)	Peak volume (SBV)	Yield (%)
STREAMLINE 25						
run 1	0.74	4.71	19.7	12	1.1	90
run 2	0.75	4.83	19.9	11	1.2	87
STREAMLINE 200	48	4.82	20.4	16	1.9	88
STREAMLINE 600						
run 1	420	4.87	19.9	17	2.2	87
run 2	440	4.48	20.8	11	2.1	92
STREAMLINE 1200	1640	5.60	20.4	12	2.3	88

SBV = sedimented bed volumes

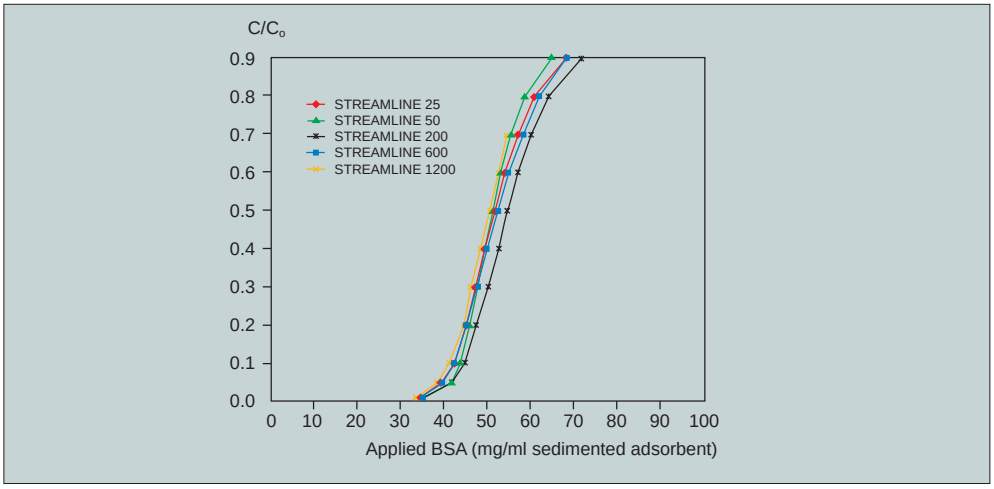


Fig. 14. Breakthrough curves for BSA on STREAMLINE DEAE from laboratory to production scale. (Work by Pharmacia Biotech.)

4. Method Optimization

Feed application

This section indicates what effect different feed-stock variables may have on the behaviour of expanded bed adsorption, and gives guidelines on suitable corrective actions to ensure stable expansion and consistent function with different types of feed material.

Viscosity

When the crude feed is pumped onto the column using the same flow rate as was used for bed expansion and equilibration, the expansion usually increases further due to the viscosity of the feed-stock being higher than the viscosity of the equilibration/expansion buffer. Very high viscosities can have a negative impact on the stability of the expanded bed. A moderate increase in viscosity does not effect bed stability but it can cause over-expansion when using nominal flow velocities of around 300 cm/h. Such a high degree of expansion causes adsorbent beads to pack tightly against the adaptor net. A packed zone of beads against the adaptor net acts as a depth filter and traps particulate material present in the crude feed, eventually blocking flow through the column. If particulates are seen building up against the adaptor net during feed application, a periodic back-flush helps remove them. A switch to downward flow eliminates the build-up after a few seconds. When the build-up has been eliminated, a switch back to upward flow quickly restabilizes the expanded bed. Viscosity is more of a concern when working with intracellular systems compared to the more dilute broths resulting from systems where the target molecule is secreted to the culture medium.

The effect of feed-stock viscosity and biomass content on expanded bed adsorption has been studied by Barnfield Frej *et al* (28) in an application to recover Annexin V from unclarified *E. coli* homogenate. Other host organisms or other experimental conditions may give different results, but the data reported by Barnfield Frej *et al* can be useful as a general guide to the effect of feed-stock characteristics on the hydrodynamic properties of an expanded bed.

To study the effect of biomass dry weight, the cell suspensions were homogenized until the viscosity was less than 10 mPa s at a shear rate of 1 s^{-1}, see Table 4.

Table 4. Example of biomass content and viscosities in *E. coli* homogenates tested on a STREAMLINE DEAE adsorbent (15 cm sedimented bed height) expanded in a STREAMLINE 50 column (5 cm i.d.; 100 cm tube height), applying a flow rate of 300 cm/h (28).

Biomass content		**Viscosity**		**No. of passages in homogenization**
Dry weight (%)	Wet weight (%)	At 1 s^{-1} shear rate (mPa s)	At 50 s^{-1} shear rate (mPa s)	At approx. 700 bar
4	14	7	3	3
5	17	8	4	4
6	21	9	5	4
7	24	15	7	7
8	27	30	15	6

Trouble-free expansion was achieved at biomass dry weights up to 5%. At higher dry weights, the bed expanded to the top of the column and caused a build-up of adsorbent beads against the adaptor net. At dry weights of 7% and above, it was not possible to reduce viscosity to below 10 mPa s by multiple passages through the homogenizer, but it was still possible to use dry weights of 7–8% if the flow direction was reversed periodically to prevent build-up against the adaptor net. No evidence of decreased bed stability, e.g. channelling in the expanded bed, could be detected at dry weights up to 8%. Dry weights higher than 8% resulted in channelling in the expanded bed and poor recovery of the target protein. At a dry weight of 9.2%, the expanded bed collapsed to close to the sedimented bed height due to heavy channelling.

Barnfield Frej *et al* also studied the effect of viscosity while keeping the biomass dry weight constant at approximately 3.4%. The viscosity was varied by passing the feed-stock three times through the homogenizer at pressures ranging from 300 to 950 bar. Trouble-free expansion was achieved at viscosities up to 10 mPa s. At viscosities above 10 mPa s, the bed expanded to the top of the column, requiring periodic reversal of flow direction to prevent build-up against the adaptor net. It was possible to use viscosities up to 50 mPas without evidence of channelling in the bed. Feed-stocks with viscosities above 50 mPa s gave rise to channelling and poor recovery of the target protein. At viscosities of 500 mPa s, the bed collapsed to close to the sedimented bed height.

These results seems to agree with results reported by Chang and Chase (34) using STREAMLINE DEAE in a STREAMLINE 50 column for purification of glucose-6-phosphate dehydrogenase from unclarified yeast cell homogenates.
They concluded that a biomass dry weight of 7% could be readily processed with no sign of bed instability if the flow rate was decreased to prevent excessive expansion of the bed.

When high biomass content and high viscosity cause frequent build-up of adsorbent against the adaptor net, the flow rate should be reduced, or the viscosity of the feed-stock decreased, to reduce bed expansion during feed-application. Reduction of viscosity can easily be accomplished by diluting the feed-stock with buffer or water. When the target protein is accumulated intracellularly, the viscosity may be reduced by further homogenization of the feed-stock. After a few runs through a high pressure homogenizer, the viscosity is usually in the order of 5 mPa s.

Nucleic acids

High viscosity can also be related to a high content of nucleic acids in the feed-stock. Treatment of the feed-stock with a nuclease, e.g. Benzonase[1], can give the desired decrease of viscosity (28). In an intracellular system, reduction of viscosity by nucleic acid degradation using a nuclease is more efficient if the nuclease is added to the cell suspension prior to, rather than after, he homogenization. This treatment enables fewer passages through a high pressure homogenizer to reach a viscosity suitable for expanded bed adsorption.

The effect of nucleic acids may be particularly severe if they originate from lysis of cells in an extracellular expression system, or from a pierced cytoplasmic membrane during release of product accumulated in the periplasmic compartment. These two cases release larger fragments of nucleic acids than does release from an intracellular expression system by normal application of high pressure homogenization for cell disintegration. The nucleic acids released from lysed cells may not have a severe effect on the viscosity of the feed, but are more likely to cause problems related to aggregation, clogging and fouling of the adsorbent. They may cause significant clogging of the inlet liquid distribution system of the column, generating increased back pressure and uneven flow distribution over the column cross-section. An uneven flow distribution in turn causes channelling in the expanded bed and reduced expansion during sample application. If the effect is less severe, it may only be seen as a moderate increase in back pressure and some slight channelling in the lower part of the expanded bed. Sometimes, nucleic acids that have entered the bed may cause formation of large aggregates in the expanded bed, which may be difficult to remove from the bed during feed application and the subsequent wash phase. Problems with clogging due to released nucleic acids can be circumvented by treating the feed material with nuclease (e.g. Benzonase) which will degrade the nucleic acids into smaller fragments (46).

---

[1] Benzonase is a genetically engineered endonuclease, produced in *E. coli,* which is active on all forms of DNA and RNA. According to information provided by the supplier (Merck, Nycomed Pharma A/S) the enzyme is produced under strict regulatory control to make it a suitable tool in industrial scale bioprocesses. The enzyme is more than 90% pure, free from viral contaminants and proteases. Any impurities derive solely from the *E. coli* host. An ELISA kit is available for validating processes where Benzonase is used.

Even if the concentration of nucleic acids in the feed is not extremely high and there is no sign of deteriorated hydrodynamic properties of the expanded bed, they may still attach to the adsorbent beads by non-specific interaction causing a slow build up which may effect binding characteristics after a number of purification cycles. Such contamination may be removed from the column by washing with a few bed volumes of 1M NaOH/1M NaCl. Complete removal may only be accomplished by treating the bed with a nuclease.

Another consequence of the presence of nucleic acids in the feed material is that they may effect the binding capacity and/or the selectivity. This is not solely related to nucleic acids but also to other types of polyionic macromolecules or highly charged insoluble material such as phospholipids, polysaccharides, cell debris or whole cells. Nucleic acids, which carry negative charges, can bind to anion exchangers and block capacity, but they may also form complexes with the target protein in cation exchange applications where the target protein is positively charged. This may cause loss in product yield since neutral protein-nucleic acid complexes show weak binding to the medium and are eluted in the flow through fraction. This type of interaction between nucleic acid and contaminating proteins in the feed material also affects selectivity during adsorption. The net effect of these interactions will depend on which adsorbent is used, the working pH and the profile of contaminating proteins in the feed material.

Aggregation of biomass

Cells and cell debris from different hosts often tend to aggregate at low pH. If this effect is severe, it blocks the column inlet distribution system. It is therefore important to test at an early stage that the conditions selected during the method scouting phase are compatible with the unclarified feed stock. When problems occur, they are usually associated with the low pH used during cation exchange chromatography. Problems can thus be circumvented by applying anion exchange chromatography instead.

Occasionally, debris may aggregate inside the expanded bed during feed application eventually blocking the column adaptor net. To prevent this type of problem, the adaptor net can be replaced by the elutriation sealing.

Cell agglomeration

The formation of cell agglomerates in the feed material is a further point of concern when working with feed material from secretion systems. This is more significant if the sample application time is long, as when applying a dilute feed at maximum loading capacity. This is because dead cells show an increased tendency to form agglomerates, and cell viability in the feed decreases rapidly when the fermentation has been terminated and the feed prepared for application to the expanded bed column. Cell agglomerates usually form in areas of stagnant liquid which makes it important to apply continuous stirring of the feed material throughout sample

application. Once cell agglomerates have formed, they serve as nuclei for the formation of larger aggregates. If they enter the column, they can partially block the inlet liquid flow distribution system, which causes increased back pressure and channelling in the lower part of the bed versus the end of the sample application. Large aggregates that cannot pass freely through the mesh screen of the column can be removed prior to application by using a simple in-line crude mesh filter. If aggregates enter the expanded bed, they can interact with the adsorbent, forming even larger aggregates that are difficult to remove from the bed during the wash phase. If this occurs, it can block the flow when the bed is to be eluted in the packed mode. As a result, it is necessary to elute the column in expanded mode. In this case, removal of aggregates from the bed by applying an efficient cleaning-in-place protocol is crucial to prevent build-up from cycle to cycle.

Cell lysis

As already discussed in Section 3, cell lysis is one of the main concerns when processing feed material from secretion systems, since it usually releases nucleic acids, lipids and other cell membrane components causing bed instability during processing and fouling of the adsorbent. This makes it more difficult to restore performance between purification cycles. In addition, release of intracellular proteases can have a negative effect on the overall yield of active product.

Cell lysis may be of special concern in ion exchange applications, since the culture broth has to be diluted and pH adjusted prior to loading of the feed-stock. Such operations must be exercised with care since shifts in pH and/or osmolality can accelerate cell lysis. Dilution buffer (to adjust pH and ionic strength) should be added immediately before adsorption to minimize exposing cells to conditions that will promote cell lysis. This is especially important when sample application time is long. It will be even more important in large scale applications, since scaling up usually increases holding times between the different operational steps. Dilution just prior to adsorption can easily be achieved by applying on-line dilution according to Fig. 15. Ideally the diluent should have a high osmolality to prevent cell lysis due to increased osmotic pressure. The osmolality of a cell culture is approximately 300 mOsm/kg. An example of a suitable diluent is a solution of 200 mM D-glucose in water giving an osmolality of approximately 200 mOsm/kg.

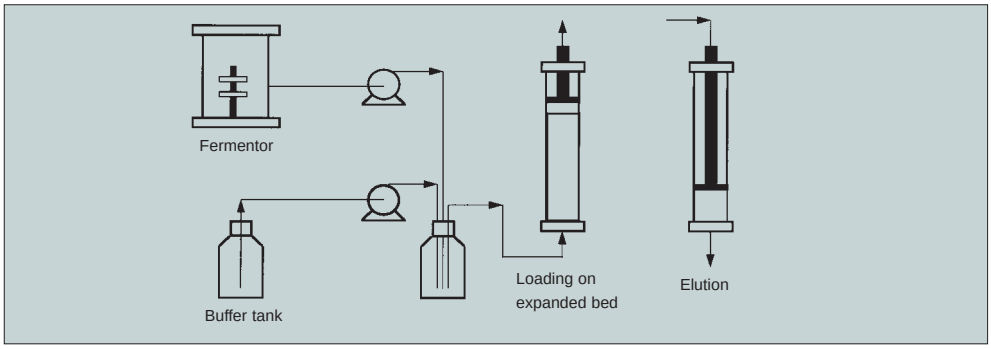


Fig. 15. Schematic diagram of process flow during adsorption with on-line dilution of the culture broth.

Wash

In any type of adsorption chromatography, the washing stage removes non-bound and weakly bound soluble contaminants from the chromatographic bed. In expanded bed adsorption, washing also removes remaining particulate material from the bed. Since expanded bed adsorption combines clarification, concentration and initial purification, the particulate removal efficiency is a critical functional parameter for the optimal utilization of the technique.

Washing is performed by pumping starting buffer through the expanded bed with upward flow until the UV-signal from the column effluent returns to close to the base line. This requires approximately 5–20 sedimented bed volumes of buffer, which will also ensure an almost complete removal of particulate material from the column. The required wash volume depends on the type of feed material used. Feed material based on secretion systems, e.g. hybridoma cell cultures, requires smaller wash volumes for complete particulate removal. The flow rate at start of the wash cycle should be the same as during feed application. The wash volume can be minimized by lowering the adaptor at start of the wash and keeping it just above the surface of the expanded bed for the remainder of the wash cycle (39). In large scale STREAMLINE columns, an adsorbent sensor is available for automatic lowering of the adaptor.

The efficiency of washing out particulate material from a STREAMLINE DEAE and STREAMLINE SP column is demonstrated in Fig. 16 (44). *E. coli* homogenates, containing approximately 10^9 cfu/ml, were applied to 250 ml media. The relative reduction of living cells was approximately 10^5 after washing with buffer for a volume corresponding to 20 sedimented bed volumes, which is in the same range as when using traditional clarification techniques. A slight increase in the number of living cells was observed in the eluate from STREAMLINE DEAE when the NaCl concentration in the eluate was increased. This is due to the fact that *E. coli* cells are negatively charged and bind to the positively charged adsorbent surface. Similar results have been reported by Hansson et al (32) and Batt et al (25). Hansson et al applied 8 litres of crude *E. coli* fermentor broth to 200 ml STREAMLINE DEAE adsorbent and reported a 4-log reduction of viable cell count after a wash with six expanded bed volumes of buffer. Batt et al applied 26 litres of crude CHO culture broth to 170 ml STREAMLINE SP adsorbent and reported an almost complete removal of suspended cells after a wash with 4 litres of buffer.

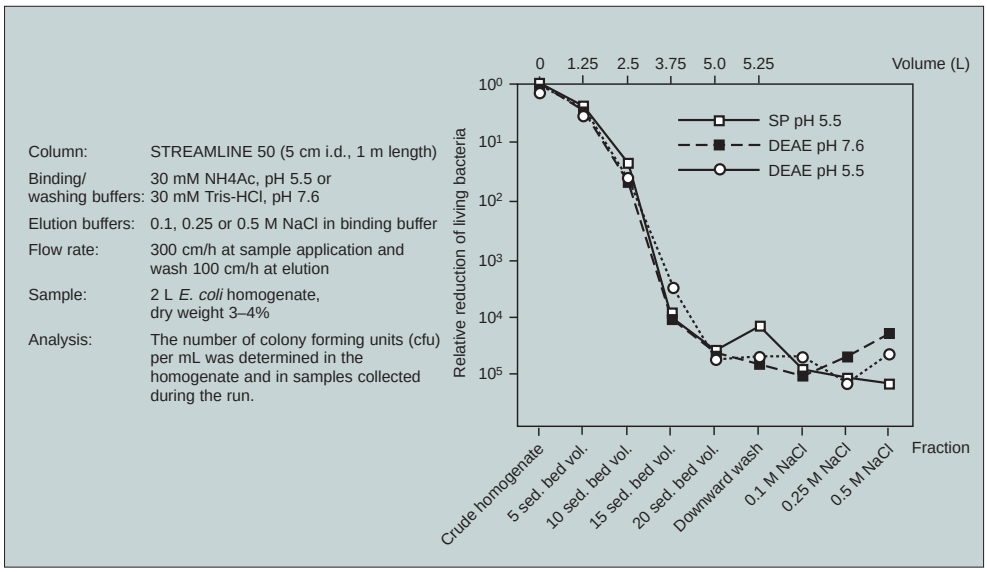

Column: STREAMLINE 50 (5 cm i.d., 1 m length)
Binding/ washing buffers: 30 mM NH4Ac, pH 5.5 or 30 mM Tris-HCl, pH 7.6
Elution buffers: 0.1, 0.25 or 0.5 M NaCl in binding buffer
Flow rate: 300 cm/h at sample application and wash 100 cm/h at elution
Sample: 2 L E. coli homogenate, dry weight 3–4%
Analysis: The number of colony forming units (cfu) per mL was determined in the homogenate and in samples collected during the run.
0 1.25 2.5 3.75 5.0 5.25 Volume (L)
Relative reduction of living bacteria
SP pH 5.5
DEAE pH 7.6
DEAE pH 5.5
Crude homogenate
5 sed. bed vol.
10 sed. bed vol.
15 sed. bed vol.
20 sed. bed vol.
Downward wash
0.1 M NaCl
0.25 M NaCl
0.5 M NaCl
Fraction

Fig. 16. Relative reduction of living *E. coli* cells during the wash and elution steps on STREAMLINE DEAE and SP at pH 5.5 and 7.6. (Work by Pharmacia Biotech.)

In an application on STREAMLINE rProtein A for purification of monoclonal antibodies (64), clarification efficiency was determined by particle analysis using a Coulter Counter (Fig. 17). A total of 60 litres of a whole hybridoma cell culture broth was applied to 150 ml of adsorbent. Wash was performed with buffer until the UV-signal returned to the base line. It was shown that after an initial retardation, all the particles contained in the feed left the column with the flow through fractions. A more than 100-fold reduction of particles was detected in the eluate fraction.

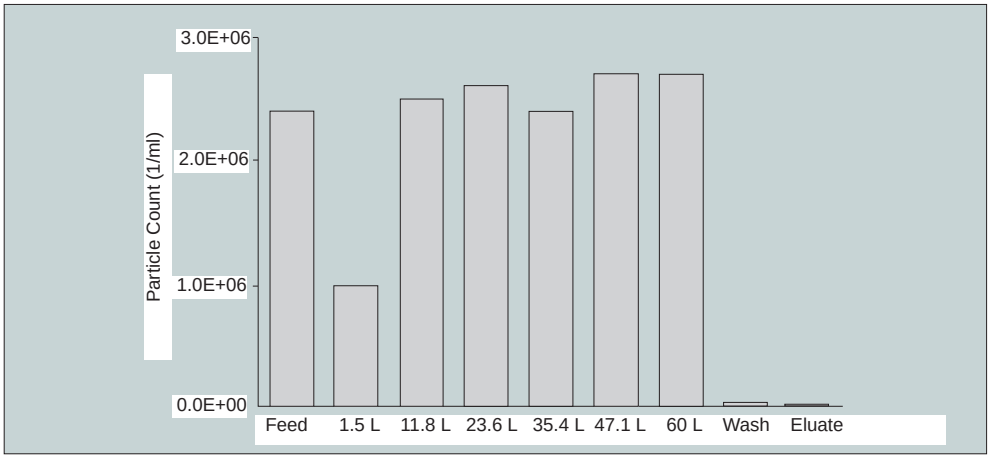


Fig. 17. Total particle concentration in different fractions from purification of a monoclonal antibody on STREAMLINE rProtein A. (Reproduced with permission from Ref. 64)

Washing may also be performed with a buffer containing a viscosity enhancer such as glycerol, which may reduce the number of bed volumes needed to clear the particulates from the bed. A viscous wash solution follows the feedstock through the bed in a plug-like manner, increasing the efficiency of particulate removal. Complete removal of particulate material by washing with one expanded bed volume of 25–50% (v/v) glycerol has been reported by Chang and Chase (34).

Even if the clarification efficiency of an expanded bed adsorption step is very high, some interaction between cell/cell debris material and adsorbent beads can be expected, which retain small amounts of cells and/or cell debris on the adsorbent. Such particulates may be removed from the bed during regeneration, for instance when running a high salt buffer through an ion exchanger, or during cleaning between cycles using a well-defined CIP protocol.

Cells retained on the adsorbent may be subjected to lysis during the washing stage. Such cell lysis can be promoted by reduced ionic strength when wash buffer is introduced into the expanded bed. Nucleic acids released due to cell lysis can cause significant aggregation and clogging owing to the "glueing" effect of nucleic acids forming networks of cells and adsorbent beads. If not corrected during the washing stage, wash volume/time may increase due to channelling in the bed. Other problems may also arise during later phases of the purification cycle, such as high back pressure during elution in packed bed mode and increased particulate content in the final product pool. If such effects are noted during washing, a modified wash procedure containing Benzonase (Merck, Nycomed Pharma A/S) can be applied to degrade and remove nucleic acids from the expanded bed.

The following protocol is an example of a suitable wash procedure for removing aggregation caused by released nucleic acids. The protocol may have to be further optimized according to the specific conditions in any particular application.

- Wash the bed with 5 sedimented bed volumes of starting buffer, upward flow, 300 cm/h.

- Wash the bed with 2 sedimented bed volumes of starting buffer containing 2 mM $MgCl_2$ and 50 µl (~13000 units) Benzonase per litre buffer, upward flow, 300 cm/h.

- Wash the bed with 8–10 sedimented bed volumes of starting buffer, upward flow, 300 cm/h.

Elution

When cells, cell debris and other particulate material have been removed from the expanded bed during the wash stage, the bed can be sedimented in the column and eluted in packed mode in exactly the same way as in packed bed chromatography. Elution in packed mode reduces the volume of eluent needed and gives a more highly concentrated product pool. It also decreases the risk of contaminating the product pool with trace amounts of cells and aggregates that may still be bound to the adsorbent beads and desorbed with the target protein during elution.

Step-wise elution is often preferred to continuous gradients since it allows the target protein to be eluted in a more concentrated form, reduces buffer consumption and gives shorter cycle times. Being a typical capture step, separation from impurities in expanded bed adsorption is usually achieved by selective binding of the product, which can simply be eluted from the column at high concentration with a single elution step.

The flow during elution can be directed either in the same direction as during sample application, i.e. upwards, or in the reverse direction, i.e. downwards. If only a small fraction of the adsorbent's capacity has been used, resulting in adsorbed material being located predominately at the inlet of the bed, reversed flow direction is likely to give a more concentrated product pool. If the maximum capacity of the adsorbent has been used, resulting in adsorbed material being located over the complete bed, or predominantly at the bed outlet due to displacement effects, elution by upward flow may be preferred.

The flow velocity during elution also affects the concentration of the product pool. A lower flow velocity will give a smaller elution volume. The optimal flow velocity for eluting proteins from STREAMLINE adsorbents is in the range of 50–150 cm/h, considering the time for elution and volume of the collected product pool.

If large aggregates are formed in the bed during application of the feed-stock, it can be difficult to remove them all during the wash stage. If such aggregates are still present in the bed at start of elution, it might be necessary to elute the column in expanded mode to avoid excessive back pressure in the column. The aggregates subsequently have to be removed from the bed during the cleaning-in-place stage. The concentration effect will also be substantial when eluting in expanded bed mode, which is demonstrated in Table 5, showing data from elution of lysozyme from STREAMLINE SP (44).

Table 5. The effect of elution mode on the volume of eluted fraction of lysozyme. Lysozyme was loaded on STREAMLINE SP at 20% of the adsorbents total capacity and eluted with a step elution procedure using 1 M NaCl in binding buffer. Elution flow rate was 100 cm/h.

Elution mode	Eluted volume (ml)	Ratio eluted volume/ sedimented bed volume
Sedimented, downward flow	308	0.99
Sedimented, upward flow	350	1.12
Expanded, upward flow	426	1.36

Elution in sedimented mode with downward flow gave the smallest elution volume. Elution in expanded mode increased the elution volume by approximately 40%. The symmetry of the eluted peaks was virtually identical, independent of elution mode.

Cleaning-in-place (CIP)

The working life of chromatographic media has a significant impact on process economy in downstream processing. A long working life means less frequent replacement of the media, resulting in decreased running costs and increased overall process economy. The working life of a STREAMLINE adsorbent is affected by the different types and amounts of contaminating material present in the feed-stock applied to the column. In any type of chromatography, precipitated, denatured or non-specifically bound substances can block binding capacity and/or interfere with the chromatographic selectivity of the adsorbent. In expanded bed adsorption, media are further challenged by the nature of the feed-stock which may contain cells, cell agglomerates, cell debris and other membrane associated particulate material as well as a high content of lipids and nucleic acids released by cell lysis. Such contamination can disturb the hydrodynamic properties of the bed by physical entrapment of large aggregates in the bed or by strong interaction causing aggregation of adsorbent beads.

The effect of such disturbances may be increased axial dispersion or even severe channelling in the bed, often accompanied by a reduced degree of expansion. Channelling and turbulence in the bed can also cause an increase in the volume of buffer needed before the UV-signal returns to baseline during the wash stage. In severe cases of fouling, the adsorbent can even form a compact "plug" in the column, when re-expansion is attempted after elution in packed bed mode. This can only be dissolved by repeated backflushing or stirring of the adsorbent in the column.

It is therefore vital to define efficient CIP protocols, designed and optimized on a case-by-case basis, to restore both hydrodynamic and chromatographic functionality between runs. Such a protocol should be applied after each chromatographic run using upward flow through the column with the adaptor positioned at a level equivalent to twice the sedimented bed height. The CIP procedure should be carried out immediately after the elution of the target protein. If adsorbent is allowed to remain in the column overnight before cleaning, it is usually more difficult to clean. Before applying the first CIP solution the bed should be expanded, and the adaptor lifted to twice the sedimented bed height, by pumping elution buffer with upward flow through the bed at a flow velocity of 100 cm/h. The flow velocity during CIP should be moderate to allow a high contact time between adsorbent beads and the cleaning agent. The volume of CIP solution should be reasonably large to allow efficient wash-out of solubilized contaminants from the bed.

The efficiency of the CIP protocol should be verified by running repetitive purification cycles and testing several functional parameters such as degree of expansion, number of theoretical plates in the expanded bed and breakthrough capacity.

Studies with different types of feed materials on different types of STREAMLINE adsorbents (29, 31, 33, 34, 45, 46, 58) have revealed that hydrodynamic and

chromatographic properties can be maintained over a large number of purification cycles if simple but efficient CIP protocols are performed between each run.

If the nature of the coupled ligand allows it, an efficient CIP protocol would be based on 0.5–1.0 M NaOH as the main cleaning agent. NaOH is the most widely accepted cleaning agent in downstream processing, since it is a cost effective technique that provides not only efficient cleaning but also sanitizes complete column systems and destroys pyrogens. It is also a safe technique, since it leaves no harmful traces in the column that can contaminate the product. If the medium to be cleaned is an ion exchange medium, the column should always be washed with a concentrated aqueous solution of a neutral salt, e.g. 1–2 M NaCl, before cleaning with NaOH. Usually this is part of the regeneration at the end of the elution stage. This removes most of the residual proteins and other contaminants attached to the medium by strong electrostatic interaction. The NaOH wash will then remove irreversibly precipitated/denatured substances and lipids.

In its simplest form, the CIP protocol can be composed of a single wash with NaOH, possibly adding 1 M NaCl to further increase the cleaning efficiency. The flow velocity should be low, e.g. 30–50 cm/h, and the volume applied should be large enough to allow a minimum contact time of 4 hours. If this does not restore the performance of the adsorbent, an even longer contact time should be tried. A combination of long contact time and a moderate consumption of cleaning solution can be applied by first directing the main peak of the material eluted by the CIP solution to waste, and then recirculating the CIP solution on the column for the remainder of the cleaning time, which can then be extended to overnight exposure or beyond. Even if the adsorbent shows a grainy appearance and poor expansion due to heavy channelling, and thus poor contact with the cleaning liquid, it slowly improves during the wash phase if the contact time is long enough. If aggregation of the bed is severe, resulting in the formation of a compact “plug” in the column, this must be eliminated by periodic back-flushing before starting the cleaning. This is done by intermittently changing the flow direction in the bed for short periods applying a high flow velocity (e.g. up to 2500 cm/h) to mechanically break up the “plug” and clumps.

If the performance of the adsorbent cannot be restored by a wash with NaOH (or NaOH/NaCl) alone, solvent- or detergent-based cleaning methods should be used in conjunction with NaOH, after having washed out the NaOH from the column with distilled water. A polar organic solvent such as 30% isopropanol or 20–70% ethanol can be a good complement to a NaOH based cleaning protocol. About three sedimented bed volumes are applied at a flow velocity of approximately 100 cm/h. If the use of organic solvents are considered less attractive due to requirements for classified areas and explosion proof equipment, a non-ionic detergent may be an alternative. Another alternative to an organic solvent may be a wash with hot water, which is a technique frequently used in the dairy industry for lipid removal. About 10 sedimented bed volumes of hot distilled water (60–95 °C) are applied at a flow velocity of 100 cm/h. A combined wash with 25% acetic acid/20% ethanol has sometimes proven to be an efficient cleaning protocol that can also be combined with an initial NaOH wash.

Occasionally, the presence of nucleic acids in the feed is the cause of fouling the adsorbent and in such a case, treating the adsorbent with a nuclease (e.g. Benzonase, Merck, Nycomed Pharma A/S) could restore performance. Benzonase can be pumped into the bed and be left standing for some hours before washing it out.

Sometimes the delicate nature of the attached ligand prevents the use of harsh chemicals such as NaOH. For instance, protein ligands such as in STREAMLINE rProtein A will hydrolyse when exposed to high concentrations of NaOH, which may limit the working life-time of the medium. Alternative cleaning agents that can be recommended in such cases are 6 M guanidine hydrochloride, 6 M urea and 1 M acetic acid. More detailed recommendations about suitable cleaning protocols can be found in the instructions accompanying each medium.

A logical approach to defining a suitable cleaning protocol can be as follows: (See also Section 5, Experimental Technique, for detailed information on how to operate the column during CIP.)

1) Set up a small scale system and run a number of purification cycles, without a CIP cycle in between, until the point is reached where it is obvious that hydrodynamic and/or chromatographic properties are compromized. Sample load should be in the same percent range as in the final production method.
2) Try to restore bed performance by cleaning with different types of agents. Start with 0.5 - 1.0 M NaOH or a mixture of 0.5 M NaOH and 1 M NaCl using a fairly long contact time, e g. 4 hours. If performance is not restored, extend the exposure time to overnight treatment. If performance is still not completely restored, try other cleaning agents, such as an organic solvent, a detergent, 25% acetic acid/20% ethanol, a chaotropic agent, etc.
3) After one or several cleaning agents have been identified for their effectivness in recovering stability and performance of the expanded bed, a suitable cleaning protocol is defined and applied in a scaled down version of the process to verify cleanability and repetitive use of the bed. The cleaning protocol should be as simple as possible. Preferably, such a study is performed on new medium and the cleaning protocol should be applied at the end of each purification cycle.

Productivity

Productivity in downstream processing is a complex issue which relates to all the different characteristics of both the feed material and the adsorbent.

In specific terms, productivity is defined as the amount of product produced per adsorbent volume and time unit (Productivity = g litre^{-1} h^{-1}).

Dynamic binding capacity

The amount of product produced per adsorbent volume (g/litre) relates to the dynamic binding capacity and yield of the target protein. Dynamic binding capacity is a function of the flow velocity through the expanded bed and the uptake rate of the target protein under defined processing conditions, i.e. pH, conductivity, viscosity etc. Due to the impact on mass transfer resistance in the system, dynamic binding capacity generally decreases with increase in flow velocity and with increase in viscosity of the feed. The effect on mass transfer by viscosity is via the effect on the diffusion coefficient, which decreases at increased viscosity. The viscosity is usually of greater significance in expanded bed adsorption than in traditional packed bed chromatography due to high biomass content and presence of particulate material in crude unclarified feed materials. Particulates and polyionic macromolecules in a raw feed may also affect dynamic binding capacity by interacting with binding sites in the adsorbent.

Dynamic binding capacity is defined by determining the breakthrough capacity for the target protein in expanded bed mode using frontal analysis. This is done at the end of the method optimization phase when the effect of the crude feed on the hydrodynamic and chromatographic performance of the expanded bed has been carefully examined and all necessary corrections have been made to ensure consistent functionality and robustness. This work also includes the final decision on what will be the most suitable flow rate to apply during feed application in expanded mode. The technique is the same as that applied during the method scouting phase using clarified feed in packed bed mode. The process feed is continously applied to the column at the defined flow velocity until breakthrough of the target protein can be detected in the column effluent. A breakthrough curve from expanded bed adsorption of a recombinant Fab-fragment on STREAMLINE SP is shown in Fig. 18. The breakthrough profile of the Fab-fragment was determined by running an ELISA on single fractions collected during feed application and wash. The concentration of the Fab-fragment in single fractions (C) was plotted in the chromatogram in relation to the concentration of the Fab-fragment in the feed applied to the column (C_o). In this specific application, the main breakthrough occurred when approximately 8 litres of feed had been applied to the column. When defining a suitable maximum loading capacity in subsequent production runs, a safety margin has to be applied to compensate for different sources of variability in the process that may affect binding capacity. In practical terms, this usually means that to avoid the risk for valuable product leaking off the column during feed application, maximum loading should be in the range of 50–75% of the loading where the main breakthrough was defined in the breakthrough study.

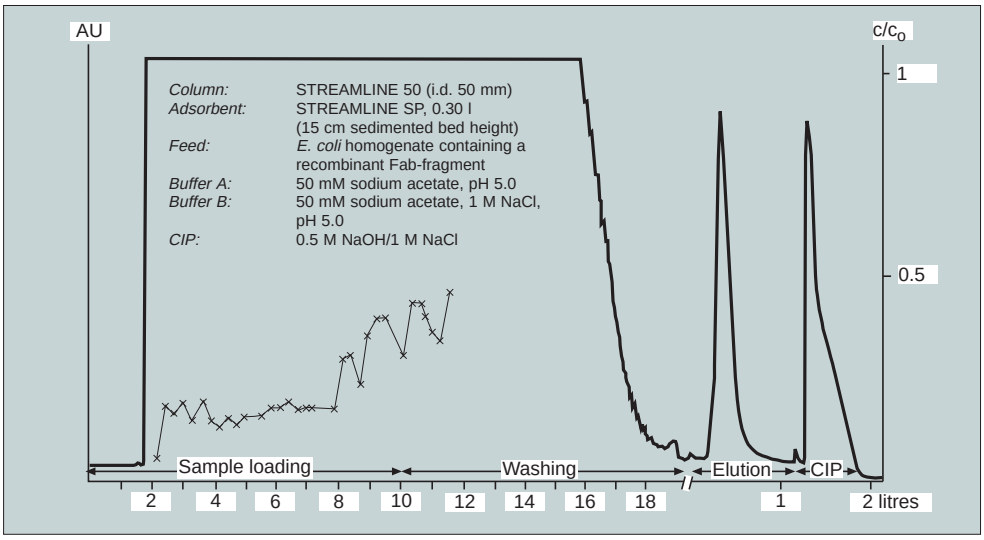


Fig. 18. Determination of breakthrough capacity for a recombinant Fab-fragment on STREAMLINE SP. (Work by Pharmacia Biotech.)

Process time

Process time is the time of a complete purification cycle, i.e. the sum of the time for expansion/equilibration, feed application, washing, elution, regeneration and cleaning-in-place.

The processing time is a function of volumetric flow rate and volume applied onto the column at each stage of the process. The sample application stage often has the most significant impact on the processing time, especially when the feed consists of an unprocessed diluted cell culture broth. A long sample application time can also have a negative effect on productivity by decreasing the yield of active product due to cell lysis releasing proteases and/or glycosidases.

The height of the expanded bed also influences processing time. In principle, a wide, short bed gives shorter processing time compared to a narrow, tall bed. This is because processing a certain volume of liquid through a certain volume of adsorbent, using a specified flow velocity, is faster if the bed is wide and short since the processing flow velocity corresponds to a higher volumetric flow rate through the system. When applying adsorption chromatography in preparative mode, the general guideline is therefore to keep the bed height as short as possible to reduce process time.

Optimizing throughput

Throughput, i.e. the amount of feed that can be applied per volume of adsorbent and time unit, is a function of capacity and speed of the purification process. As in any type of chromatography, optimization of one of these parameters can only be realized at the expense of the other. The characteristics of the feed and the anticipated final scale of operation form the basis for the balance between capacity and speed in any particular application.

High speed may be required to reduce sample application time, particularly if cell lysis occurs, releasing destructive nucleic acids, proteases, glycosidases, etc. In practical terms, it may be important to apply high volumetric flow rates even if this means that a somewhat larger bed volume is needed for processing of a specific amount of feed. In expanded bed adsorption, the maximum flow velocity through the bed is limited by its effect on the degree of expansion and bed stability. Nominal flow velocity with STREAMLINE media is around 300 cm/h at room temperature, giving a degree of expansion of around 3 times with normal aqueous based buffers. Flow velocities significantly higher or lower than this can negativly effect the stability of the expanded bed. Application of a viscous feed to the column further increases the degree of expansion. If viscosity is significant, a flow velocity of 300 cm/h could be enough to cause the bed to expand up to the position of the adaptor, forcing the beads up against the adaptor net. Hence, it will be difficult to inrease speed in an expanded bed system by increasing flow velocity. However, sample application time can be reduced by over-sizing the column, i.e. using a wider column with the same sedimented bed height. This gives a higher volumetric flow rate at preserved flow velocity through the bed.

When processing time is not considered an important issue, optimization could be focused on reducing the scale of work, i.e. utilizing the available binding capacity of the adsorbent to its maximum. The adsorption in an expanded bed is a process which is controlled by the residence time of the target protein in the column. The residence time is the bed height divided by the flow velocity applied during feed application. Hence, the residence time can be increased by decreasing flow velocity or by increasing the bed height.

The effect of bed height on dynamic binding capacity is demonstrated in Fig. 19. The early breakthrough observed at a sedimented bed height of 5 cm is partly due to the inherent instability (i.e. back mixing) in the lower part of an expanded bed, but also due to a short residence time. It can also be explained by a decrease in the number of theoretical plates (mass transfer units), which lowers the efficiency of the adsorption process. When the sedimented bed height is increased to 10 cm, the bed stabilizes, which, together with the increased number of mass transfer units, causes a significant increase in dynamic binding capacity. A further increase in sedimented bed height is accompanied by a further gradual increase in dynamic binding capacity as the capacity approaches the total available binding capacity of the bed. A sedimented bed height of at least 10 cm is required to achieve stable expansion.

A sedimented bed height of 15 cm is recommended as a starting point for method development to avoid the risk of bed instability and to assure a reasonable number of mass transfer units. Increasing the sedimented bed height significantly above 15 cm in an attempt to further increase binding capacity, may not be an attractive approach in expanded bed adsorption. This is because it can cause the bed to expand up to the point where adsorbent beads start to pack against the adaptor net, especially when a highly viscous feed is applied. Increased sedimented bed height also gives increased cycle time.

Increasing residence time by decreasing flow velocity may have a negative effect on bed stability, due to decreased expansion. However, it may be possible to decrease flow velocity without compromising the degree of expansion since the viscosity is usually significantly higher in the feed than in the equilibration buffer. How significant the effect of this is depends on the resistance to mass transfer in the system. It may be significant for a high molecular weight protein, especially if the viscosity in the feed is high and slows down molecular diffusion. This approach was taken by Chang and Chase (23, 34) who noticed a 2.5-fold increase in dynamic binding capacity when applying a viscous feed containing lysozyme to STREAMLINE SP at a reduced flow velocity that was continously controlled to keep the degree of expansion constant (twice the sedimented bed height) during the entire process.

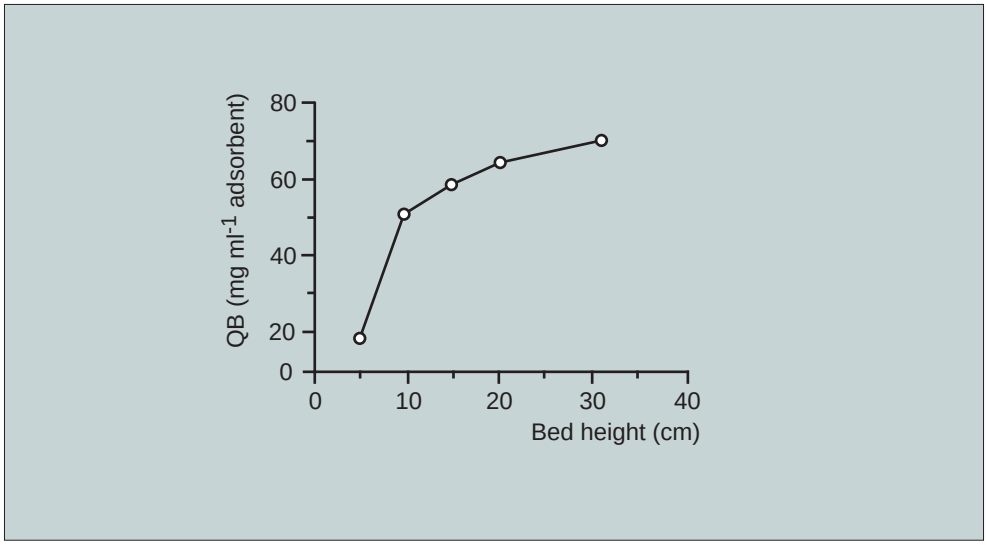


Fig. 19. Effect of sedimented bed height on breakthrough capacity (QB) for lysozyme on STREAMLINE SP. (Work by Pharmacia Biotech.)

5. Experimental Technique

Chapter 5 examines the practical aspects of expanded bed adsorption. It shows examples of different system set-ups and describes their operation in detail.

System configurations

This section describes how to set up a system for expanded bed adsorption. It covers system configurations and selecting components for both manual and automatic modes. The instructions for start-up and system operation refer to a manual or semi-automated system as outlined in figures 20 and 21.

Manual systems

A STREAMLINE column is easily set up for manual operation. It requires two pumps, manual valves, and UV, conductivity and pH monitors.

One of the pumps controls the adaptor movement by pumping hydraulic liquid into the hydraulic chamber of the STREAMLINE column. The other pumps liquid through the column. The most suitable type of pump for expanded bed adsorption is a peristaltic pump. The advantage of peristaltic pumping is that the crude feed does not contaminate the pump, making stripping and cleaning unnecessary. The pumping action is also reasonably gentle, which minimizes cell lysis when pumping whole cell culture broth. The limited tolerance to back pressure during peristaltic pumping is not a problem since expanded bed adsorption systems generate only low pressure.

One double channel valve is needed to reverse flow through the column. A number of single channel valves are needed to select buffer or feed at the inlet side, collect product at the outlet side, by-pass the column, and control adaptor movement by the hydraulic pump.

A pressure monitor can be installed before the column bottom valve. This gives an early indication on any pressure build-up in the system. Pressure can build-up if the column nets are clogged or if adsorbent beads start packing against the adaptor net. This can occasionally happen during application of a crude and viscous feed and may require periodic reversal of flow, reduction of flow rate or reduction of viscosity of the feedstock.

Fig. 20 shows a basic set up of a manual STREAMLINE system.

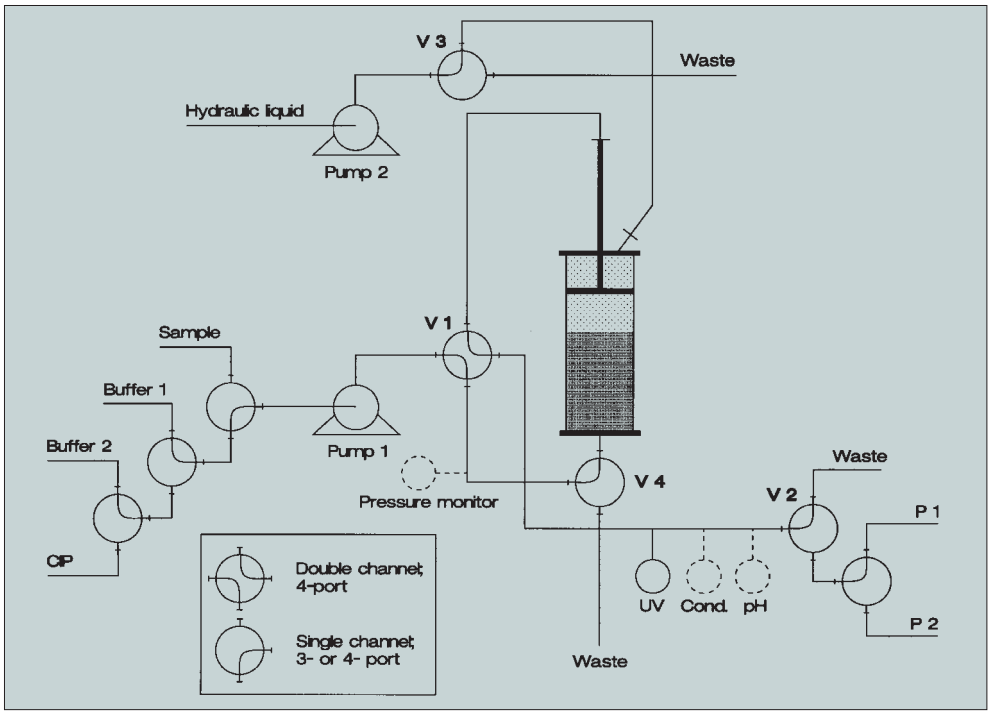


Fig. 20. Schematic representation of a manual STREAMLINE system with two pumps.

Valve V1 changes the direction of flow from upward to downward when eluting bound substances from the sedimented bed. It can also be used to reverse flow intermittently to prevent the column nets clogging or adsorbent beads building-up under the adaptor end plate during feed application. This valve may also be needed to dissolve possible plug formation in the bed prior to cleaning-in-place.

Valve V2 directs effluent to the product collection vessel during elution. It also blocks the flow through the bed to allow the adaptor to be lifted in the column when Pump 1 pumps upward flow through the bed.

Valve V3 directs the flow of hydraulic liquid from the hydraulic chamber to waste when the adaptor is lifted. It also opens the flow path from Pump 2 to the hydraulic chamber of the column when the adaptor is lowered. Valve V3 is closed during normal use with upward or downward flow through the bed (see Fig. 24). Alternatively, valve V3 can be positioned to open the flow path from the hydraulic chamber to waste. This will allow the adaptor to move upwards in case the adaptor net is clogged and thus prevent pressure build-up in the system. This is useful if no pressure monitor is connected to the column inlet. If valve V3 is a 4-port valve, the fourth port must be blocked.

Valve V4 is a column bottom valve used to by-pass the column when filling the system at the inlet side. The bottom valve also allows a column containing sedimented adsorbent to be connected or disconnected without being drained.

Table 6 shows valve positions and pump modes used to direct flow and control adaptor movement.

Table 7 lists recommended equipment for assembling complete manual systems based on STREAMLINE 25, STREAMLINE 50 and STREAMLINE 200 columns.

Table 6. Pump mode and valve positions for directing flow and controlling adaptor movement in a manual/semi-manual STREAMLINE system as outlined in Fig. 20 and Fig. 21.

Operation	**Valve 1** (double channel, 4-port)	**Valve 2** (single channel, 3- or 4-port)	**Valve 3** (single channel, 3- or 4-port)	**Pump 1**	**Pump 2**
Upward flow				on	off
Downward flow				on	off
Adaptor up				on	off
Adaptor down				off	on
Adaptor down/ Upward flow				on	on

Table 7. Components recommended for a manual STREAMLINE system at different scales of operation. See "Ordering Information" for descriptions, pack sizes and code numbers.

Component	STREAMLINE 25	STREAMLINE 50	STREAMLINE 200
Valves Double channel	SRV-4	4-way; 1/4" i.d.; PP[1] 4-way; 6 mm i.d.; SS[2]	4-way; 1/2" i.d.; SS[1] 4-way; 10 mm i.d.; SS[2]
Single channel	SRV-3	L-type; 1/4" i.d.; PP[1] L-type; 6 mm i.d.; SS[2]	L-type; 1/2" i.d.; SS[1] L-type; 10 mm i.d.; SS[2]
Tubing	1.9 x 2.7 mm; PTFE	1/4" i.d.; PE[1] 6 mm i.d.; PVC[2]	1/2" i.d.; PE[1] 10 mm i.d.; PVC[2]
Pumps	Watson Marlow 504 U/RL (Rotation speed: 220 rpm)	Watson Marlow 504 U/RL (Rotation speed: 220 rpm)	Watson Marlow 604 U/R (Rotation speed: 165 rpm)
Peristaltic tubing [3]	1.6 mm i.d.	3.2 mm i.d.	9.6 mm i.d.
Connectors	2.7 mm o.d. (M6)	1/4" i.d. (JACO 10-4-2)[1] (valve connectors)	1/2" i.d. (JACO 10-8-6)[1] (valve connectors)
Unions	25 mm o.d. clamp to M6 [4]	25 mm o.d. clamp to 1/4" threaded [5]	25 mm o.d. clamp to 1/2" threaded [5]
UV-monitor/Flow cell	UV-1/S2	UV-1/Industrial 6 mm i. d.[2]	UV-1/Industrial 10 mm i.d.[2]
Recorder	REC 102	REC 102	REC 102
Miscellaneous	Stop plug Plastic clamp 25 mm Gasket 6 mm i.d.	Blind flange and packing Plastic clamp 25 mm Gasket 6 mm i.d.	Blind flange and packing Plastic clamp 25 mm Gasket 10 mm i.d.

1 Threaded connections.

2 25 mm o.d. clamp connections.

3 Peristaltic tubing ordered from Pharmacia Biotech can be supplied with moulded on 25 mm o.d. clamp connectors as an option if sanitary connections are required.

4 For connection to peristaltic tubing supplied with 25 mm o.d. clamp connectors.

5 For connection of PE tubing (threaded polyethylene tubing) to column, peristaltic tubing and UV flow cell.

Semi-automated systems

A small scale semi-automated system can be set up with the STREAMLINE 25 column and GradiFrac, a fraction collector with built-in control of flow rate, three switch valves, and recorder start and stop. Fig. 21 shows a schematic representation of this configuration. The system shown in Fig. 21 contains one STREAMLINE 25 column and one extra column for traditional packed bed chromatography. More columns can be connected if more valves are added to the system.

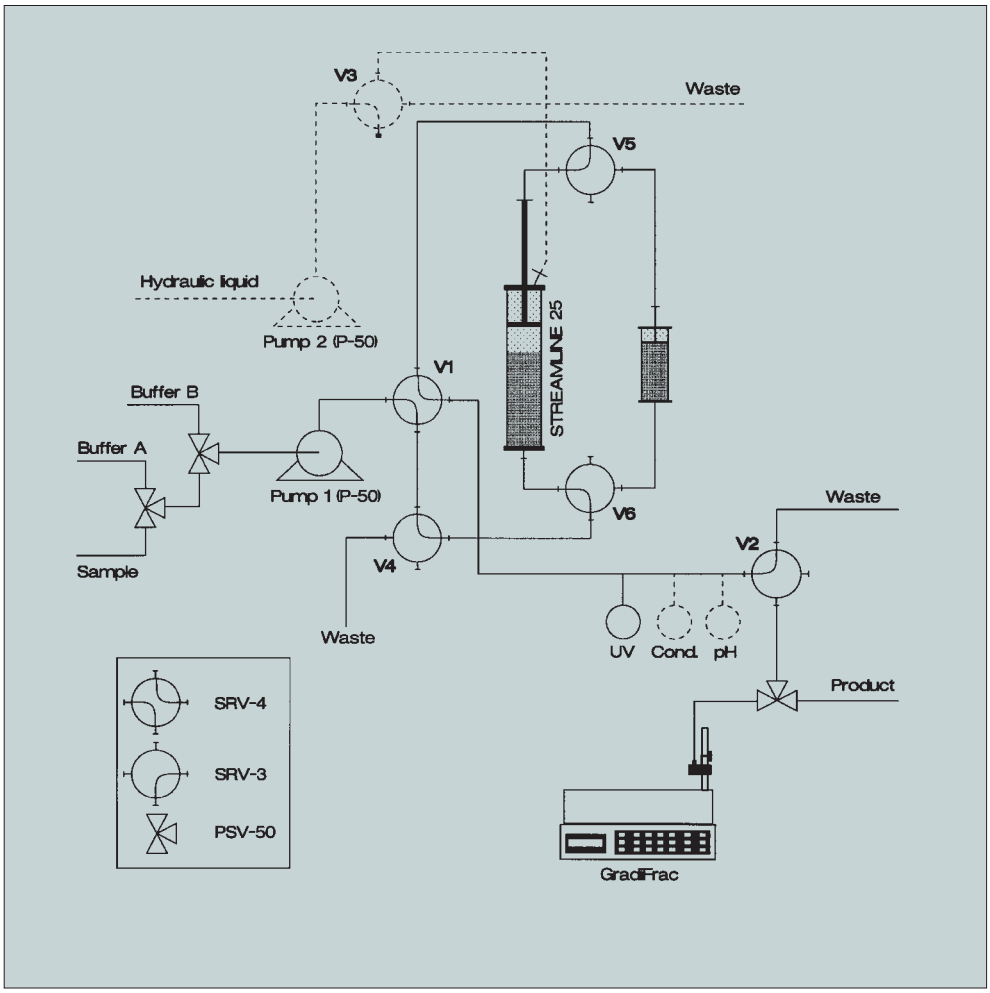


Fig. 21. Schematic representation of a semi-automatic STREAMLINE system based on the STREAMLINE 25 column and GradiFrac fraction collector.

Pump 1 and the PSV-50 valves are controlled by a programmed sequence in the GradiFrac controller. Valves V1, V2, V3, V4, V5 and V6 are manual valves.

Valve V1 changes flow direction through the columns connected to the system.

Valve V2 directs the column effluent to the fraction collector. It also blocks the flow through the bed to allow the adaptor to be lifted when pump 1 pumps upward flow through the bed.

Valve V3 directs the flow of hydraulic liquid from the hydraulic chamber to waste when the adaptor is lifted. It also opens the flow path from pump 2 to the hydraulic chamber of the column when the adaptor is lowered. Valve V3 should be closed during normal upward or downward flow through the bed (see Fig. 21).

Alternatively, it can be left in its waste position to allow for upward movement of the adaptor in case of build-up of pressure drop over the adaptor net. The fourth port of the valve has to be blocked.

(Valve V3 and pump 2 are optional. They are needed to operate a STREAMLINE 25 column supplied with a hydraulic adaptor, but are not required if the STREAMLINE 25 column is supplied with a manual adaptor.)

Valve V4 by-passes columns connected to the system.

Valves V5 and V6 are used to select individual columns connected to the system.

Table 6 shows valve positions and pump modes used to direct flow and control adaptor movement.

Table 8 lists components suitable for assembling a complete semi-automated system based on STREAMLINE 25 and GradiFrac.

Table 8. Components for a semi-automated STREAMLINE system based on a STREAMLINE 25 column and GradiFrac. See "Ordering Information" for descriptions, pack sizes and code numbers.

Fraction collector/Controller	GradiFrac
Manual valves	
Double channel	SRV-4
Single channel	SRV-3
Solenoid valves	PSV-50
Pump	P-50
Tubing	1.9 x 2.7 mm; PTFE
UV-monitor/Flow cell	UV-1/S2
Connectors	2.7 mm o.d. (M6)
Recorder	REC 101/102
Miscellaneous	Rack

Automated systems

Small scale operation with ÄKTAexplorer

STREAMLINE 25 column may be connected to ÄKTAexplorer chromatography system for completely automated method optimization and small scale processing.

Re-configure the ÄKTAexplorer system as described below. (See Fig. 22 for the complete system configuration.)

1) Disconnect the prefilter and the mixer.

2) Connect Pump A directly to flow direction valve (V7) position 7.

3) Disconnect the sample loop and the sample pump from the injection valve (V1).

4) Connect Pump B directly to injection valve position 2.

5) Connect injection valve position 3 to the hydraulic inlet of the STREAMLINE column.

6) Install 1.0 mm i.d. tubing in the flow path after the column. By-pass the pH flow cell and the flow restrictor to reduce system back pressure in the column.

7) Fit a stop plug in outlet valve (V4) position 8.

Setting the injection valve to position 2 and the outlet valve to position 8 opens the flow path from the hydraulic chamber of the column to W1 and blocks the column outlet. This lifts the adaptor in the column when pump A pumps upward flow through the bed.

The adaptor is lowered by pumping hydraulic liquid via pump B into the hydraulic chamber. Open the flow path from pump B to the hydraulic inlet by switching the injection valve to position 1.

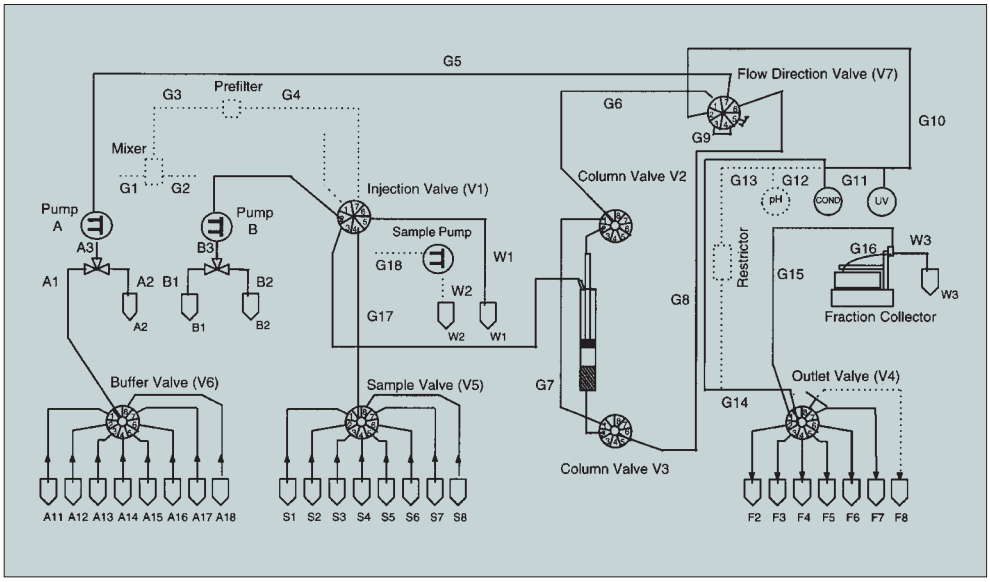


Fig. 22. Schematic representation of ÄKTAexplorer system reconfigured for expanded bed adsorption chromatography using a STREAMLINE 25 column.

Small scale operation with BioPilot System

The STREAMLINE 25 column can also be connected to BioPilot System for completely automated control of expanded bed adsorption chromatography at small scale.

Re-configure BioPilot System as described below. (See Fig. 23 for the complete system configuration.)

1) Disconnect Superloop from Valve 1 position 2. (It is not required.)

2) Connect tubing between Valve 1 position 2 and the column hydraulic inlet.

3) Disconnect the tubing from Valve 1 position 1 and connect it to a SRTC-3 connector.

4) Connect tubing between the SRTC-3 connector and Valve 1 position 1.

5) Connect tubing between outlet W1 and the third port on the SRTC-3 connector.

6) Direct tubing from outlet W2 to the container for hydraulic liquid.

7) Fit a stop plug in Valve 6 position 8.

8) Remove the filter from the Prefilter 6000 between pump A and the mixer.

The flow path from pump A to the expanded bed will be open whenever Valve 1 is in positions 1 or 3. When Valve 1 is in position 3, the flow path from the hydraulic chamber of the column to the hydraulic liquid container will also be open.

Setting Valve 1 to position 3 and Valve 6 to position 8 blocks the column outlet and lifts the adaptor by upward flow through the column.

The adaptor is lowered by pumping hydraulic liquid via pump C into the hydraulic chamber. To open the flow path from pump C to the hydraulic inlet, valve 1 should be in position 1.

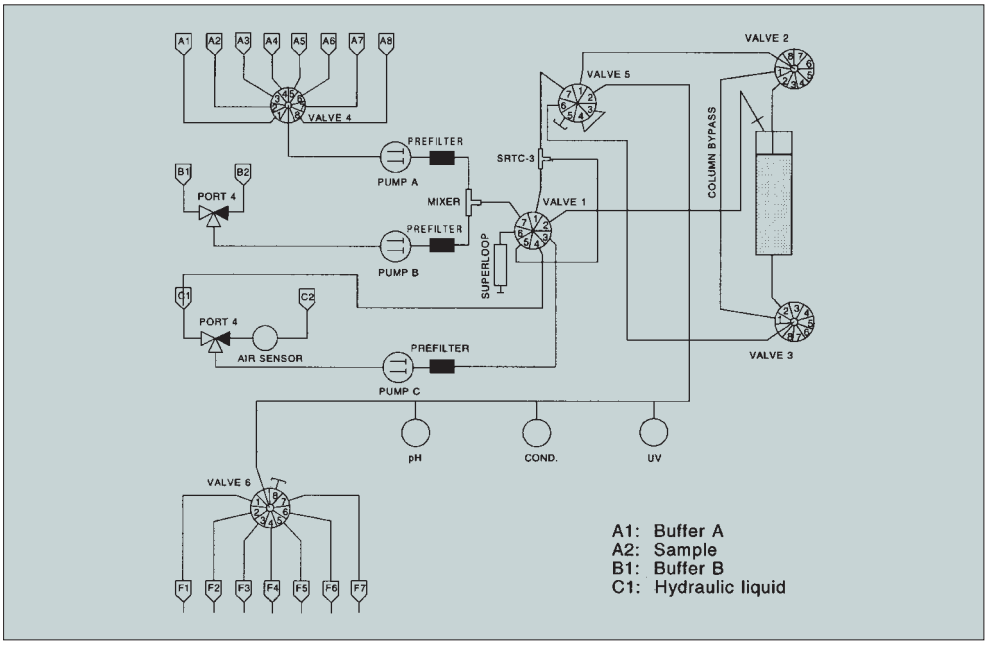


Fig. 23. Schematic representation of a BioPilot system reconfigured for expanded bed adsorption chromatography using a STREAMLINE 25 column.

Pilot scale/production scale

Pharmacia Biotech supplies both manual and fully automated STREAMLINE systems in a range of scales. Systems based on STREAMLINE 50 and STREAMLINE 200 columns are suitable for method development and small scale production. Full scale production systems based on STREAMLINE CD (custom designed) columns have a capacity of several hundred litres sedimented bed volume.

See Chapter 6 "Product Guide" for more information about large scale STREAMLINE columns and systems.

Start-up

This section describes the preparation required prior to an expanded bed adsorption separation. Detailed information about assembling and operating STREAMLINE columns and components is given in the individual column Instruction Manuals.

Sampling the adsorbent

Each STREAMLINE adsorbent contains particles with a wide range of bead size and density. When taking a sample of adsorbent from the container, take great care to ensure that the beads represent this range in both size and density.

Gently shake the container until the adsorbent is completely suspended. Then immediately pour all the adsorbent into a glass filter funnel (porosity #3) or a bucket, depending on the amount. Allow the adsorbent to drain*, cut out a triangular slice of the cake and weigh it.

Calculate the amount of adsorbent needed for a specific height of sedimented bed in the STREAMLINE column:

Amount of drained adsorbent (g) = density of adsorbent cake (g/ml) x sedimented bed height (cm) x cross sectional area of the column (cm^2).

The approximate density of the adsorbent cake is 1.2 g/ml for STREAMLINE DEAE, STREAMLINE SP, STREAMLINE Q XL, STREAMLINE SP XL, STREAMLINE Chelating and STREAMLINE Heparin. For STREAMLINE rProtein A, the approximate density of the adsorbent cake is 1.3 g/ml.

Connecting the column

Remove the adaptor and connect the column to the system via the column bottom valve as shown in Fig. 20. Fill the column approximately 2/3 full with distilled water via the pump and bottom valve. While pumping through the bottom valve, suck out any air that may be trapped under the column end-piece net with tubing connected to water suction or a peristaltic pump. Move the tubing over the whole of the upper surface of the end-piece net. Leave about 5 cm of water in the column.

*To drain adsorbent in a bucket, connect one end of a piece of tubing containing a filter to water suction and use it to suck away the excess liquid (the filter prevents loss of adsorbent). Move the tubing very gently over the sedimented adsorbent surface. Handle the drained adsorbent carefully to avoid damaging the adsorbent particles.

Loading adsorbent

Suspend the adsorbent in starting buffer to give an approximate 50% slurry. Without allowing the adsorbent to sediment, quickly pour the slurry into the column. Wash out any remaining adsorbent from the container with buffer and pour this into the column. Be careful not to trap air in the slurry. Make sure that no aggregates of air-adsorbent remain floating on the liquid surface. Fill the column to the rim with distilled water.

Inserting the adaptor

Insert the assembled adaptor into the column at an angle so that one side of the adaptor net is in the water-filled column. Without trapping air under the net, carefully put the adaptor into a vertical position. Slowly push the adaptor down until the gasket on the adaptor net is submerged in water. This ensures that the gasket forms a tight seal with the glass tube. Fill the space above the adaptor with distilled water so that the hydraulic drive can function. Push down the lid and secure it in place.

For the STREAMLINE 25 column, insert the adaptor into the column without force so that the adaptor O-ring rests on top of the glass tube. Fill the space above the adaptor with distilled water so that the hydraulic drive can function. Push down the lid and secure it in place.

Switch valve V3 to open the flow path from pump 2 to the hydraulic chamber of the column. Set valve V2 to waste position to open the column outlet. Slowly move the adaptor down by pumping hydraulic liquid from the hydraulic pump into the hydraulic chamber of the column. Stop the hydraulic pump when the adaptor has been lowered a few centimeters. Switch valve V3 to open the flow path from the hydraulic chamber to waste. Set valve V2 to the closed position to block the flow path through the bed. Lift the adaptor by pumping buffer with upward flow from pump 1 to remove any air trapped in the hydraulic chamber. If necessary, repeat this procedure until all air has been removed from the column hydraulics.

Position the adaptor at a height corresponding to approximately four times the height of the sedimented bed to allow for bed expansion.

Set valve V3 to its closed position (see Fig. 24) and valve V2 to its waste position.

Filling the system

Set the column bottom valve (V4) to the by-pass position. Start pump 1 and fill all tubing with the correct buffer by switching the appropriate valves on the inlet side. Fill the sample inlet tubing with starting buffer. Make sure that all air has been removed from tubings and valves. Increase the flow rate in the system to force out any remaining air bubbles. While pumping equilibration buffer through the system, put the column in-line by switching valve V4 to direct the flow to the bottom column inlet.

System operation

Expansion/Equilibration

1) Check that the stand base is mounted vertically. Use a spirit level to check that the column is vertical. Adjust if necessary and secure the stand feet or wheels.

Note: A vertical column is crucial for optimal results.

2) Mark the height of the sedimented bed on the column tube. Before the first run, the bed must be expanded and sedimented in the column to be able to determine the correct sedimented bed height. The sedimented bed height is used to determine the degree of expansion when expansion and equilibration is completed.

3) Position the valves according to Fig. 24. Start pumping equilibration buffer through the column with upward flow at the pre-determined feed application flow velocity.

4) Allow the bed to stabilize at this flow velocity for approximately 30-40 minutes. The bed is stable when no further expansion can be observed and only small circular movements of the individual particles are visible (see Fig. 8).

5) When the pH/conductivity of the outlet stream is the same as the equilibration buffer, recirculate the buffer by connecting the system outlet to the equilibration buffer container. Continue until test sample or feed is applied to the bed.

6) Check the stability of the expanded bed using the test principles outlined in "Evaluation of bed stability" in Chapter 2.

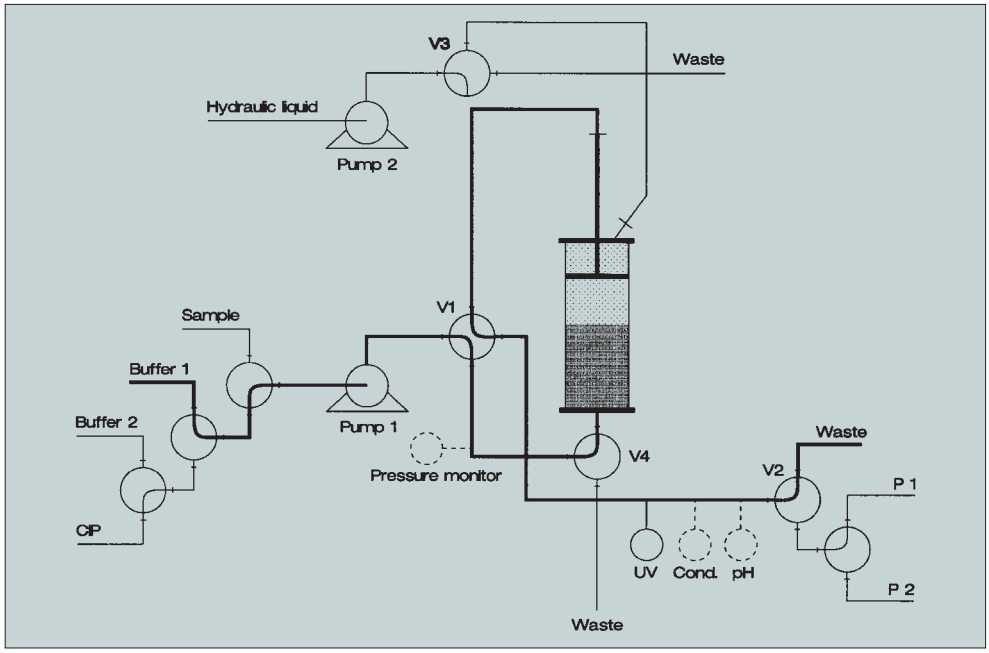


Fig. 24. Schematic representation of valve positions and liquid flow in a manual STREAMLINE system during expansion/equilibration.

Feed application

1) Position the adaptor 3-4 cm below the top of the column tube to allow for the bed expanding, which usually occurs when feed is applied. When the behaviour of the feed-stock is better known, the adaptor can be positioned 5-10 cm above the height to which the bed expands during feed-stock application.
2) When the expanded bed is stable and equilibrated with adsorption buffer, switch to feed application as shown in Fig. 25. Stir the feed-stock continuously during application to prevent aggregates forming.
3) Continuously monitor the level of the expanded bed. The degree of expansion usually increases during this phase due to the increase in viscosity from the crude feed.
4) Periodically back-flush to clear the adaptor net if particulates build up underneath it due to increased bed expansion. Switch valve V1 to downward flow to disrupt this build-up. Return to upward flow after a few seconds when the build-up has been cleared. The expanded bed quickly re-stabilizes.

   Decrease expansion by reducing the flow velocity if build-up is observed frequently. If high biomass content and high viscosity cause bed instability and channelling, decrease viscosity by diluting the feed-stock. Refer to "Feed application", in Chapter 4 "Method Optimization" for more details about the effect of feed material on bed stability and performance.

   Valve V3 can be put in its waste position during feed application and wash, especially if there is no pressure monitor connected to the system. This will allow the adaptor to move upwards in case of build-up of pressure drop over the adaptor net.

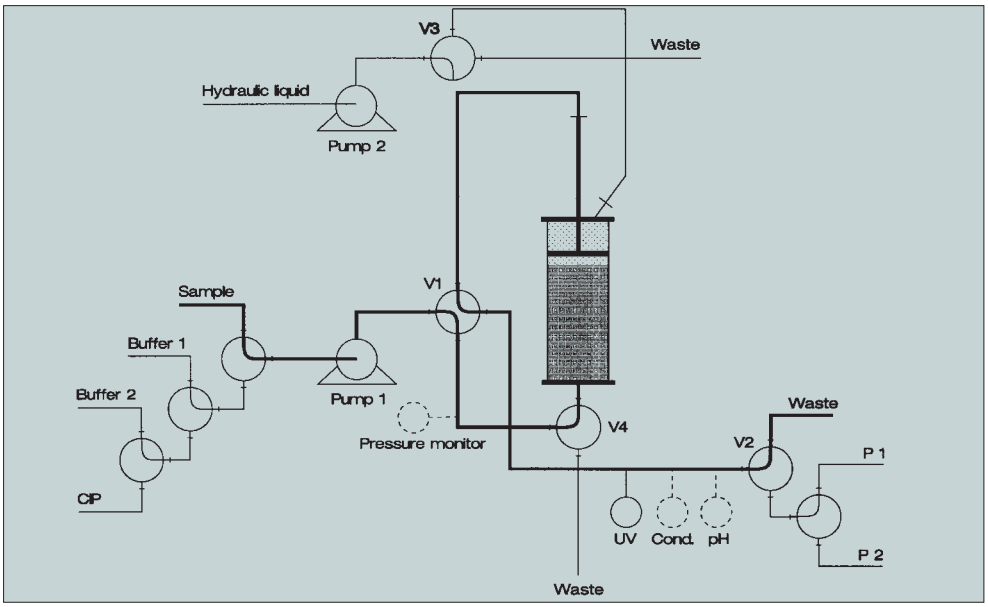


Fig. 25. Schematic representation of valve positions and liquid flow in a manual STREAMLINE system during feed application.

Wash

1) When all the sample has been applied, switch to wash buffer and use upward flow in expanded mode to remove loosely bound material and cells. Use the same flow velocity as that used during feed application.

2) While washing at this flow velocity, lower the adaptor to just above the surface of the expanded bed (see Fig. 26). This speeds up the wash cycle and reduces the consumption of wash buffer. This effect will be further enhanced by repeatedly adjusting the adaptor to keep it just above the surface of the expanded bed for the remainder of the wash. If the feed-stock contains a lot of particulates, wait until most have been flushed out before lowering the adaptor. Particulates can be trapped in the net if the adaptor is lowered too early or too quickly.

3) Continue washing until the signal from the UV monitor returns to the base line.

4) Switch to downward flow for a few seconds, and then back to upward flow. Repeat this procedure several times. This back-flush removes any particulates that might still be trapped in the distribution system.

5) Turn off the pump and allow the bed to sediment.

6) When the adsorbent has sedimented, move the adaptor down towards the surface. Stop when the edge of the adaptor net touches the bed.

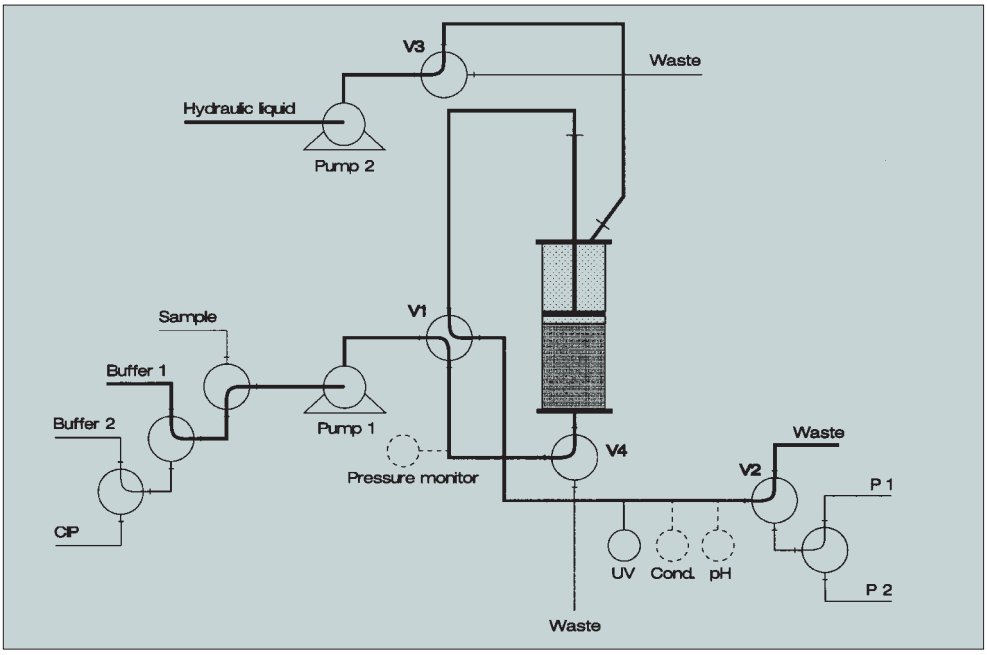


Fig. 26. Schematic representation of valve positions and liquid flow in a manual STREAMLINE system during washing. The figure shows valve positions when lowering the adaptor while maintaining upward flow through the expanded bed. The adaptor is lowered by pumping hydraulic liquid from pump 2 into the hydraulic compartment of the column.

Elution

1) Switch valve V1 to downward flow and start pumping 1–2 sedimented bed volumes of wash buffer through the sedimented bed at a flow velocity of approximately 100 cm/h.

2) Switch to elution buffer and continue pumping in the same direction to elute the target protein from the sedimented bed. Pump at a flow velocity of 50–100 cm/h (see Fig. 27).

3) Collect eluted fractions as indicated by the UV monitor.

4) Turn off the pump and immediately start cleaning-in-place (CIP).

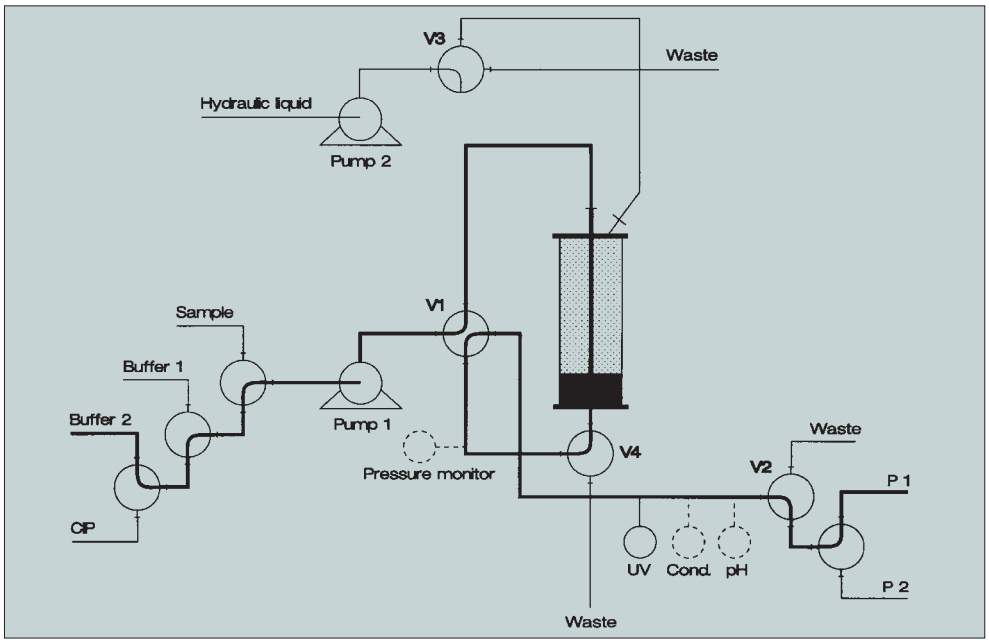


Fig. 27. Schematic representation of valve positions and liquid flow in a manual STREAMLINE system during elution.

Cleaning-in-place

Design and optimize the cleaning-in-place protocol according to the properties of the adsorbent ligand and the nature of the feed material applied. The general operating principles of cleaning-in-place an expanded bed adsorption column are described below. More detailed information about designing cleaning-in-place protocols is given under Cleaning-in-place in Chapter 4 “Method Optimization”. Specific recommendations for different types of STREAMLINE adsorbents are included in the instructions accompanying each medium.

1) Switch valve V1 to upward flow. Switch valve V3 to open the flow path from the hydraulic chamber to waste. Set valve V2 to the closed position to block the flow through the bed.

2) Lift the adaptor by pumping elution buffer with pump 1 at a flow rate of 100 cm/h.

3) When the adaptor reaches a level equivalent to twice the sedimented bed height, stop the adaptor by setting valve V2 to the waste position and valve V3 to its closed position.

4) If there is any tendency for plug formation in the bed at this stage, reverse flow to dissolve the plugs. Switch valve V1 to downward flow and return to upward flow after a few seconds. Repeat this procedure until the plugs have been broken up.

5) Continue cleaning the bed by pumping cleaning-in-place solution with upward flow through the column according the pre-defined cleaning-in-place protocol.

Maintenance

Storage

To store STREAMLINE adsorbent in the column, pump at least 5 sedimented bed volumes of storage solution through the sedimented bed at a flow rate of 100 cm/h using upward flow. Specific recommendations about storage solutions for different types of STREAMLINE adsorbents are included in the instructions accompanying each medium.

Position the adaptor on top of the sedimented bed.

Remove the lid from the column and suck out any liquid remaining in the space above the adaptor. Add 20% ethanol before replacing the lid.

Elutriation

Even if STREAMLINE adsorbents show high mechanical stability (43), elutriation may sometimes be needed to prevent the accumulation of adsorbent fines generated by repeated operation or handling of the adsorbent. These fines are washed out from the column/adsorbent using an elutriation procedure where the adaptor net has been replaced with an elutriation sealing.

This procedure is described in the Instruction Manual accompanying each STREAMLINE column.

Replacing the adsorbent

Resuspend the adsorbent in buffer. Handle the sedimented adsorbent carefully to avoid damage by shear forces. Either pour out the adsorbent slurry from the column or siphon it off.

6. Product Guide

Pharmacia Biotech supplies a complete range of STREAMLINE products from method development at laboratory scale up to full scale, routine production. This Chapter describes the different STREAMLINE adsorbents, columns and systems. Basic product characteristics and technical data are included. For information about pack sizes and code numbers, see "Ordering Information".

STREAMLINE adsorbents

Pharmacia Biotech manufactures a range of STREAMLINE adsorbents for ion exchange and affinity expanded bed adsorption. Table 9 summarizes this product range. All adsorbents are available in both laboratory pack sizes and bulk quantities.

Table 9. STREAMLINE adsorbents available from Pharmacia Biotech.

STREAMLINE SP	Strong cation exchanger
STREAMLINE DEAE	Weak anion exchanger
STREAMLINE SP XL	High capacity, strong cation exchanger
STREAMLINE Q XL	High capacity, strong anion exchanger
STREAMLINE rProtein A	Affinity adsorbent for purification of monoclonal and polyclonal antibodies
STREAMLINE Chelating[1]	Coupled iminodiacetic acid groups for immobilized metal affinity chromatography (IMAC)
STREAMLINE Heparin[1]	Coupled heparin for purification of plasma proteins (e.g. coagulation factors) and other types of proteins

1) STREAMLINE Chelating and STREAMLINE Heparin are currently (March 1997) available as CDM products, see page 61. (CDM = Custom Designed Media).

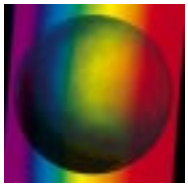
BioProcess Media

BioProcess Media are a full range of separation media specially designed, manufactured and supported to meet the demands of industrial scale biomolecule production.

This symbol is your guarantee of:

- Secure long term supply of large batches of media.
- Comprehensive documentation and regulatory support to assist in process validation.
- Conformance to the ISO 9001 Quality system for reliable supply with high quality and high batch-to-batch consistency.
- High chemical stability to allow efficient cleaning and sanitization regimes.
- Scaleable performance from bench top to production hall.
- Compatible large scale columns and equipment.

Media in Chapter 6 that fulfill the above criteria are given the BioProcess Media symbol.



Custom Designed Media

Custom Designed Media (CDM) meet the needs of specific industrial process separations when chromatography media from our standard product range are not suitable. Custom Designed Media comprise media for both packed bed chromatography and expanded bed adsorption. They can be made to BioProcess Media specifications if required. The Custom Designed Media group at Pharmacia Biotech works in close collaboration with the customer to design, manufacture, test and deliver media for specialized separation requirements. Several CDM products are also available to the general market. Media first produced as Custom Designed Media have often proven so successful that they have subsequently been introduced as BioProcess grade Pharmacia Biotech products. Custom Designed Media in Chapter 6 are given the CDM symbol.

Product availability

Contact your nearest Pharmacia Biotech office for further details of CDM products and services.

Base matrices

The base matrix in STREAMLINE media is a highly cross-linked beaded agarose derivative based on 6% or 4% agarose. This has been modified by including an inert core to provide the required density for stable expansion at high flow rates. The macrostructure of the agarose is composed of polysaccharide chains arranged in bundles that are further strengthened by inter-chain cross-linking. The resulting macroporous structure combines high physical and chemical stability with good capacities for large target molecules. Agarose-based matrices are also well appreciated for their low non-specific adsorption of macromolecules and well-documented industrial CIP protocols.

The reduction in porosity as a result of incorporating core material is insignificant for most applications.

Derivatization chemistry

Ligands are coupled to the STREAMLINE adsorbent with epoxy chemistry, which provides stable ether/thioether linkages. Fig. 28 shows the structures of the coupled ligands.

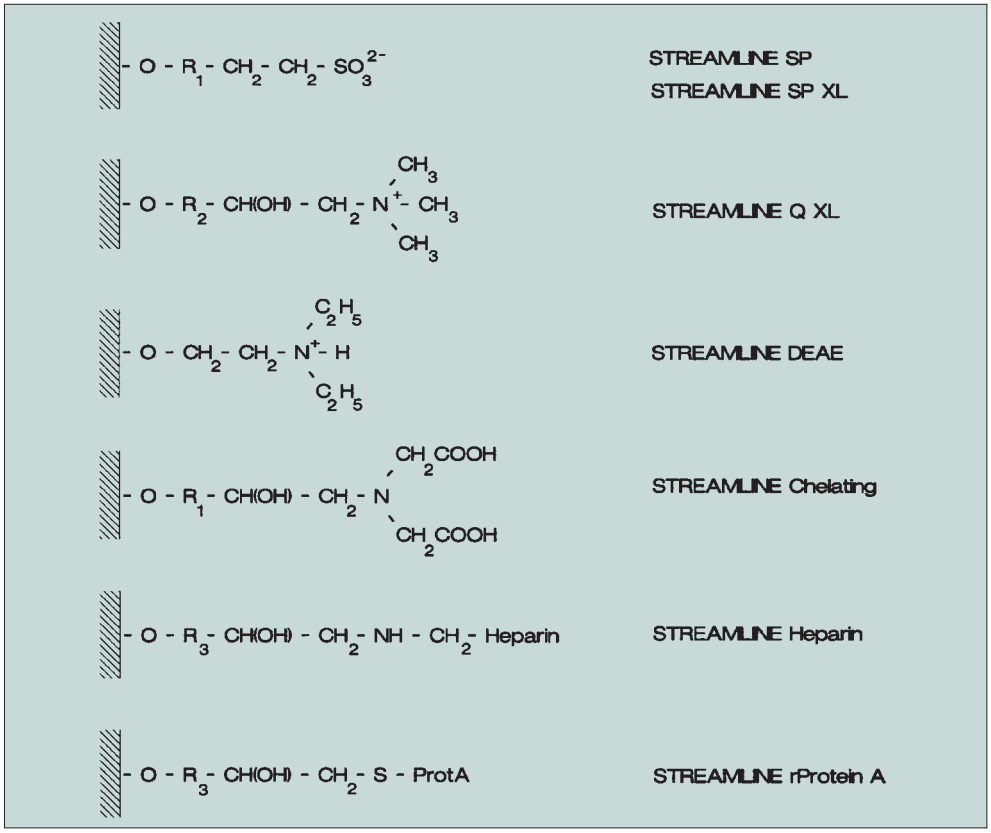


Fig. 28. The structures of coupled ligands in STREAMLINE adsorbents.

Chemical stability

STREAMLINE adsorbents are stable in all commonly used aqueous buffers, detergents, organic solvents (e.g. 70% ethanol, 30% isopropanol), and chaotropes or dissociating agents (e.g. 6 M guanidine hydrochloride and 6 M urea) commonly used to operate, clean and sanitize chromatography columns in downstream processing.

Extremes of pH, e.g. high concentrations of sodium hydroxide, are often used for cleaning, depyrogenation, virus inactivation and sanitizing columns. The pH stability of STREAMLINE adsorbents varies according to the stability of the ligands attached to the base matrix. STREAMLINE ion exchangers and STREAMLINE Chelating are substituted with small synthetic ligands and can be exposed to 1 M NaOH. In contrast, biological ligands such as protein A and heparin are susceptible to hydrolysis at extremes of pH. Care must therefore be taken when cleaning and sanitizing these media to avoid decomposing the attached ligands and significantly shortening their working life.

Specific information about pH stability is found in the description of each STREAMLINE adsorbent.

STREAMLINE adsorbents, with the exception of STREAMLINE rProtein A, can also be repeatedly sterilized by autoclaving (e.g. at 121 °C for 30 minutes).

Mechanical stability

The mechanical stability of STREAMLINE adsorbents is very high. This has been verified in both batch and expansion experiments with STREAMLINE ion exchangers (43).

In the batch experiments, a 50% slurry of the adsorbent was subjected to different types of treatment and the particle then size analysed. Table 10 shows the results as the fraction of particles smaller than 125 µm. Note that grinding effects, such as those produced by magnetic stirrers, should be avoided.

Table 10. Fragmentation of STREAMLINE adsorbent particles as a result of different types of physical treatment.

Treatment	d_n % < 125 µm
Untreated	9
Dried and rehydrated	9
Propeller mixer (high speed)	9
Peristaltic pump (twice)	14
Magnetic stirrer (30 minutes)	62

In the expansion experiments, the mechanical stability of the beads was tested by abruptly increasing the flow rate from zero to 320 cm/h, thus causing extreme turbulence in the bed. This procedure was repeated 140 times, passing a total volume of 800 litres of water (= 4000 bed volumes) through the bed over a period of 14 days. Particles leaving the column during the expansion phase were collected and their volume was found to be less than 0.2% of the total. The degree of expansion was unaffected.

Binding capacity

The porosities of STREAMLINE base matrices and the coupling densities of the ligands attached provide high binding capacities for biological macromolecules. This ensures high throughput and high productivity. As in any type of adsorption, the binding capacity for a specific target molecule depends not only on the inherent properties of the adsorbent and the target molecule, but also on the type and extent of impurities in the crude feed applied to the column.

Binding capacity data for selected model proteins are given under the description of each STREAMLINE adsorbent.

STREAMLINE SP
STREAMLINE DEAE

Product characteristics

STREAMLINE SP and STREAMLINE DEAE are ion exchange adsorbents for expanded bed mode. Both are based on highly cross-linked 6% agarose modified by including an inert quartz core to give the desired density.

STREAMLINE SP is a strong cation exchanger. The sulphonate groups maintain full protein binding capacity over the entire long term pH stability range of 4–13.

STREAMLINE DEAE is a weak anion exchanger. The number of ligand groups that are charged varies with pH. This adsorbent maintains consistently high capacities over the pH range of 2–9.

More information about STREAMLINE SP and STREAMLINE DEAE, including instructions for their use, is available in Data File STREAMLINE SP/STREAMLINE DEAE (Code No. 18-1111-73).

Table 11 summarizes their characteristics.

Table 11. Characteristics of STREAMLINE SP and STREAMLINE DEAE adsorbents.

Product	STREAMLINE SP	STREAMLINE DEAE
Type of ion exchanger	strong cation	weak anion
Total ionic capacity (mmol/ml gel)	0.17–0.24	0.13–0.21
Particle size range (µm)	100–300	100–300
Approx. mean particle size (µm)	200	200
Approx. mean particle density (g/ml)	1.2	1.2
Degree of expansion (H/H_0) at 300 cm/h	2–3	2–3
pH stability[1]		
long term	4–13	2–13
short term	3–14	2–14
Binding capacity[2] (mg/ml gel)		
lysozyme (MW 14 500)	>60	n.d.
BSA (MW 67 000)	n.d.	>40
Storage	0.2 M sodium acetate in 20% ethanol	20% ethanol

Further information is available in Data File STREAMLINE SP/STREAMLINE DEAE (Code No. 18-1111-73).

n.d. = not determined

1 Long term refers to the pH interval where the gel is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration and cleaning procedures.

2 Breakthrough capacity determined in a STREAMLINE 50 column at a flow velocity of 300 cm/h using a 2.0 mg/ml solution of protein in 50 mM sodium phosphate buffer, pH 7.5 (lysozyme) and 50 mM Tris-HCl buffer, pH 7.5 (BSA). Sedimented bed height was 15 cm.

Cleaning-in-place, sanitization-in-place and sterilization

The properties of the base matrix, the derivatization chemistry used when attaching ligands, and the inherent stability of the ligand groups, result in very stable ion exchange media. This high product stability allows exposure to harsh conditions such as 1 M NaOH for cleaning-in-place and sanitization.

Suitable cleaning-in-place protocols must be defined on a case-by-case basis depending on the nature of the feed applied to the expanded bed. Chapter 4 “Method Optimization” contains general information about cleaning-in-place of STREAMLINE adsorbents.

The following protocols developed for STREAMLINE SP and STREAMLINE DEAE restore both hydrodynamic and chromatographic properties over a large number of purification cycles using different types of *E. coli* homogenates (45).

CIP procedure 1

- 0.5 M NaOH containing 1 M NaCl, flow velocity 30 cm/h, contact time 4 hours.
- distilled water, flow velocity 100 cm/h, 3 sedimented bed volumes.
- 30% isopropanol, flow velocity 100 cm/h, 3 sedimented bed volumes.
- 25% acetic acid, flow velocity 100 cm/h, 3 sedimented bed volumes.
- equilibration buffer, flow velocity 100 cm/h, 5–10 sedimented bed volumes.

CIP procedure 2

- 0.5 M NaOH containing 1 M NaCl, flow velocity 30 cm/h, contact time 4 hours.
- distilled water (room temp.), flow velocity 100 cm/h, 3 sedimented bed volumes.
- distilled water (85-95 °C), flow velocity 100 cm/h, 10 sedimented bed volumes.
- 25% acetic acid/20% ethanol, flow velocity 100 cm/h, 1 sedimented bed volume.
- equilibration buffer, flow velocity 100 cm/h, 5-10 sedimented bed volumes.

CIP procedure 3

- 1% (w/v) DARACLEAN[1] 8471, flow velocity 30 cm/h, contact time 4 hours.
- equilibration buffer, flow velocity 100 cm/h, 5-10 sedimented bed volumes.

Procedures 1 and 2 also provide a good sanitization effect. Washing with 0.5–1.0 M NaOH for a contact time of 30–60 minutes is an effective disinfectant treatment for vegetative bacteria, yeast and moulds (47).

STREAMLINE SP and STREAMLINE DEAE can be sterilized by autoclaving the adsorbent at 121 °C for 30 minutes.

Storage

We recommend storing STREAMLINE DEAE and STREAMLINE SP in 20% ethanol. During long term storage of STREAMLINE SP in unbuffered ethanol, a gradual acidification of the storage solution may occur due to the acidic nature of the ligand. We therefore recommend adding sodium acetate up to a concentration of 0.2 M.

As an alternative storage solution for both STREAMLINE SP and STREAMLINE DEAE, we recommend 10 mM NaOH. This is comparable to 20% ethanol from a bacteriostatic point of view.

1 DARACLEAN (Grace Dearborn Ltd) is a commercially available cleaning agent containing caustic soda, alkaline salts and the non-ionic detergent Triton CF 10.

Product availability

STREAMLINE SP and STREAMLINE DEAE are supplied as suspensions in packs of 100 ml, 300 ml, 7.5 litres and 60 litres. For larger quantities, please contact your local Pharmacia Biotech office.

STREAMLINE SP is supplied in 20% ethanol containing 0.2 M sodium acetate. STREAMLINE DEAE is supplied in 20% ethanol.

STREAMLINE SP XL
STREAMLINE Q XL

Product characteristics

STREAMLINE SP XL and STREAMLINE Q XL are two high capacity ion exchange adsorbents for expanded bed mode. Their extremely high loading capacities increase the productivity of manufacturing operations.

STREAMLINE SP XL is a strong cation exchanger. The sulphonate groups maintain full protein binding capacity over the entire long term pH stability range of 4–13.

STREAMLINE Q XL is a strong anion exchanger. The quaternary amine groups maintain full protein binding capacity over the entire long term pH stability range of 2–12.

Both are based on highly cross-linked 6% agarose modified by including an inert quartz core to give the desired density. Long molecules of dextran are coupled to the agarose matrix and the strong Q and SP ion exchange groups are then attached to these dextrans chains through chemically stable ether bonds. This will cause an increase in the effective interacting volume as well as in the steric availability of the ligands for the substance to be adsorbed. The dynamic binding capacity of STREAMLINE SP XL and STREAMLINE Q XL is thus extremely high. Typical dynamic binding capacities of STREAMLINE SP XL for lysozyme in a STREAMLINE 25 column at 15 cm sedimented bed height and flow velocities of 400 cm/h are 190-220 mg/ml adsorbent.

The high dynamic binding capacities obtained with STREAMLINE XL ion exchangers can improve throughput and productivity in different ways. Speed can be increased by allowing a higher feed application flow rate for binding a certain amount of target protein to a certain amount of adsorbent. Speed can also be increased by allowing a higher conductivity during adsorption of the target protein. The crude feed may then not have to be diluted before application to the expanded bed. This smaller volume reduces feed application time. Productivity can also be improved by using a smaller amount of adsorbent for binding a certain amount of target protein, i.e. reducing the scale of work.

Table 12 compares dynamic binding capacities of STREAMLINE SP and STREAMLINE DEAE with STREAMLINE SP XL and STREAMLINE Q XL.

Table 12. Comparison of dynamic binding capacities of STREAMLINE SP/DEAE and STREAMLINE SP/Q XL for different molecules.

	STREAMLINE SP (mg/ml adsorbent)	STREAMLINE SP XL (mg/ml adsorbent)	STREAMLINE DEAE (mg/ml adsorbent)	STREAMLINE Q XL (mg/ml adsorbent)
hIgG	12	60	-	-
BSA	-	-	39	149
Ovalbumin	-	-	38	143
Lysozyme	78	213	-	-

Loading capacity at 10% breakthrough determined in a STREAMLINE 25 column at a flow velocity of 400 cm/h for STREAMLINE SP/Q XL and 300 cm/h for STREAMLINE SP/DEAE, using a 2 mg/ml solution of protein in 50 mM Tris/HCl, pH 7.5 (BSA and ovalbumin), 50 mM Glycine, pH 9.0 (lysozyme) and 50 mM sodium acetate, pH 5.0 (hIgG). Sedimented bed height was 15 cm. (Work by Pharmacia Biotech.)

More information about STREAMLINE SP XL and STREAMLINE Q XL, including instructions for their use, is available in Data File STREAMLINE SP XL/STREAMLINE Q XL (Code No. 18-1123-81).

Table 13 summarizes their characteristics.

Table 13. Characteristics of STREAMLINE SP XL and STREAMLINE Q XL adsorbents.

Product	**STREAMLINE SP XL**	**STREAMLINE Q XL**
Type of ion exchanger	strong cation	strong anion
Total ionic capacity (mmol/ml gel)	0.18–0.24	0.23–0.33
Particle size range (µm)	100–300	100–300
Approx. mean particle size (µm)	200	200
Approx. mean particle density (g/ml)	1.2	1.2
Degree of expansion (H/H0) at 300 cm/h	2–3	2–3
pH stability[1]		
long term	4–13	2–12
short term	3–14	2–14
Recommended working flow velocity (cm/h)	300–500	300–500
Binding capacity[2] (mg/ml gel)		
lysozyme (MW 14 500)	>140	n.d.
BSA (MW 67 000)	n.d.	>110
Storage	0.2 M sodium acetate in 20% ethanol	20% ethanol

Further information is available in Data File STREAMLINE SP XL/STREAMLINE Q XL (Code No. 18-1123-81).
n.d. = not determined

[1] Long term refers to the pH interval where the gel is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration and cleaning procedures.

[2] Breakthrough capacity determined in a 4.4 ml packed bed at a flow velocity of 300 cm/h using a 2.0 mg/ml solution of protein in 50 mM Glycine-HCl buffer, pH 9.0 (lysozyme) and 50 mM Tris-HCl buffer, pH 7.5 (BSA). Bed height was 10 cm.

Cleaning-in-place, sanitization-in-place and sterilization

The properties of the base matrix, the derivatization chemistry used when attaching spacers and ligands, and the inherent stability of the ligand groups, result in very stable ion exchange media. This high product stability allows exposure to harsh conditions such as 1 M NaOH for cleaning-in-place and sanitization.

Suitable cleaning-in-place protocols must be defined on a case-by-case basis depending on the nature of the feed applied to the expanded bed. Chapter 4 "Method Optimization" contains general information about cleaning-in-place of STREAMLINE adsorbents.

The protocols previously described for STREAMLINE SP and STREAMLINE DEAE adsorbents can also be applied for STREAMLINE SP XL and STREAMLINE Q XL.

STREAMLINE SP XL and STREAMLINE Q XL can be sanitized by washing the bed with 0.5–1.0 M NaOH for a contact time of 30–60 minutes. This is an effective disinfectant treatment for vegetative bacteria, yeast and moulds (47).

Storage

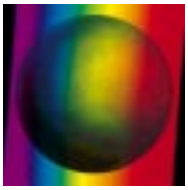
We recommend storing STREAMLINE SP XL and STREAMLINE Q XL in 20% ethanol. During long term storage of STREAMLINE SP XL in unbuffered ethanol, a gradual acidification of the storage solution may occur due to the acidic nature of the ligand. We therefore recommend adding sodium acetate up to a concentration of 0.2 M.

As an alternative storage solution for both STREAMLINE SP XL and STREAMLINE Q XL, we recommend 10 mM NaOH. This is comparable to 20% ethanol from a bacteriostatic point of view.

Product availability

STREAMLINE SP XL and STREAMLINE Q XL are supplied as suspensions in packs of 100 ml, 300 ml and 7.5 litres. For larger quantities, please contact your local Pharmacia Biotech office.

STREAMLINE SP XL is supplied in 20% ethanol containing 0.2 M sodium acetate. STREAMLINE Q XL is supplied in 20% ethanol.



STREAMLINE Chelating

Product characteristics

STREAMLINE Chelating is an expanded bed adsorbent for immobilized metal affinity chromatography (IMAC). IMAC separates proteins and peptides on the basis of their affinity for metal ions immobilized by chelation (48, 49, 50). Certain amino acids (e.g. histidine, cysteine) form complexes with these chelated metal ions around neutral pH. It is mainly the histidine content of a protein that is responsible for binding, which makes IMAC an excellent method for purifying recombinant proteins with poly-histidine fusions as well as many natural proteins.

STREAMLINE Chelating may also be used as a weak cation exchanger due to the negative charge of the deprotonated form of the carboxylic acid groups. When used as an ion exchanger, a significant part of the carboxylic acid groups lose their negative charge below pH 6. The total ionic capacity of STREAMLINE Chelating adsorbent used as a weak cation exchanger is expected to be 60-70 µmole Na^+/ml gel, which is lower than STREAMLINE ion exchange media. Despite this, STREAMLINE Chelating used as a cation exchanger may provide an alternative selectivity to the STREAMLINE ion exchange adsorbents.

STREAMLINE Chelating is based on highly cross-linked 6% agarose which has been modified by including an inert quartz core to give the desired density. It is substituted with iminodiacetic acid groups on spacer arms coupled to the STREAMLINE matrix via stable covalent linkages.

STREAMLINE Chelating is a Custom Designed Media (CDM) product. See CDM products on page 61.

More information about STREAMLINE Chelating, including instructions for use, is available in Data File “STREAMLINE Chelating”.

Table 14 summarizes the characteristics of STREAMLINE Chelating.

Table 14. Characteristics of STREAMLINE Chelating.

Chelating group	Iminodiacetic acid
Total capacity	~40 µmol Cu^{2+}/ml gel
Particle size range	100–300 µm
Approx. mean particle size	200 µm
Approx. mean particle density	1.2 g/ml
Degree of expansion (H/H0) at 300 cm/h	2–3
pH stability[1]	
long term	3–13
short term	2–14
Storage	20% ethanol

Further information is available in Data File STREAMLINE Chelating.

[1] Long term refers to the pH interval where the gel is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration and cleaning procedures.

Cleaning-in-place, sanitization-in-place and sterilization

The properties of the base matrix, the derivatization chemistry used when attaching the ligand, and the inherent stability of the ligand group, result in a very stable product. This high stability allows exposure to harsh conditions such as 1 M NaOH for cleaning-in-place and sanitization.

Suitable cleaning-in-place protocols must be defined on a case-by-case basis depending on the nature of the feed applied to the expanded bed. Chapter 4 "Method Optimization" contains general information about cleaning-in-place of STREAMLINE adsorbents.

The protocols previously described for STREAMLINE ion exchange adsorbents can also be applied for STREAMLINE Chelating.

STREAMLINE Chelating can be sanitized by washing the bed with 0.5–1.0 M NaOH for a contact time of 30-60 minutes. This is an effective disinfectant treatment for vegetative bacteria, yeast and moulds (47).

STREAMLINE Chelating can be sterilized by autoclaving the adsorbent at 121 °C for 30 minutes.

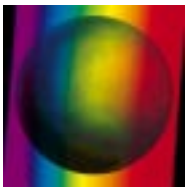
Storage

We recommend storing STREAMLINE Chelating in 20% ethanol.

As an alternative, we recommend 10 mM NaOH, which is comparable to 20% ethanol from a bacteriostatic point of view.

Product availability

STREAMLINE Chelating is supplied as a suspension in 20% ethanol in packs of 300 ml and 7.5 litres. For larger quantities, please contact your local Pharmacia Biotech office.



STREAMLINE Heparin

Product characteristics

STREAMLINE Heparin is an expanded bed affinity adsorbent for affinity chromatography using immobilized heparin. Heparin is a highly sulphated glycosaminoglycan that binds a wide range of biomolecules (51). Among the protein classes successfully purified on immobilized heparin are enzymes (mast cell proteases, lipoprotein lipase, coagulation enzymes, superoxide dismutase), serine protease inhibitors (antithrombin III, protease nexins), growth factors (fibroblast growth factor, Schwann cell growth factor, endothelial cell growth factor), extracellular matrix proteins (fibronectin, vitronectin, laminin, thrombospondin, collagens), nucleic acid-binding proteins (initiation factors, elongation factors, restriction endonucleases, DNA ligase, DNA and RNA polymerases), hormone receptors (oestrogen and androgen receptors) and lipoproteins.

STREAMLINE Heparin is based on highly cross-linked 6% agarose modified by including an inert quartz core to give the desired density. Heparin is immobilized to the STREAMLINE matrix by reductive amination, giving a stable coupling even under very alkaline conditions.

STREAMLINE Heparin is a Custom Designed Media (CDM) product. See CDM products on page 61.

More information about STREAMLINE Heparin, including instructions for use, is available in Data File “STREAMLINE Heparin”.

Table 15 summarizes the characteristics of STREAMLINE Heparin.

Table 15. Characteristics of STREAMLINE Heparin.

Ligand density[1]	~4 mg heparin/ml gel
Particle size range	100–300 µm
Approx. mean particle size	200 µm
Approx. mean particle density	1.2 g/ml
Degree of expansion (H/H0) at 300 cm/h	2–3
pH stability[2]	
long term	4–12
short term	4–13
Storage	20% ethanol

Further information is available in Data File STREAMLINE Heparin

1 The heparin used for immobilization is isolated from porcine intestinal mucosa and has a molecular weight distribution over the range 5000–30000.

2 Long term refers to the pH interval where the gel is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration and cleaning procedures.

Cleaning-in-place, sanitization-in-place and sterilization

The pH stability of STREAMLINE Heparin is limited by the susceptibility to hydrolysis of the heparin ligand at extremes of pH. However, up to 0.1 M NaOH can be used in cleaning and sanitization without significant loss of binding capacity. Alternative treatments for removing strongly adsorbed, precipitated or denatured proteins are washing with 6 M guanidine hydrochloride or 8 M urea. Non-ionic detergents at a concentration of 0.1-0.5% can be used to remove hydrophobically bound substances. Chapter 4 "Method Optimization" contains general information about cleaning-in-place of STREAMLINE adsorbents.

STREAMLINE Heparin can be sanitized by washing the bed with a mixture of 0.1 M NaOH and 20% ethanol for a contact time of 1 hour. An alternative procedure is to equilibrate the bed with 70% ethanol and allow it to stand for 12 hours. Note that specific regulations for classified areas and explosion-proof equipment may apply when handling large volumes of organic solvents.

STREAMLINE Heparin can be sterilized by autoclaving the adsorbent at 121 °C for 30 minutes.

Storage

We recommend storing STREAMLINE Heparin in 20% ethanol.

Product availability

STREAMLINE Heparin is supplied as a suspension in 20% ethanol in packs of 75 ml, 300 ml, and 7.5 litres. For larger quantities, please contact your local Pharmacia Biotech office.

STREAMLINE rProtein A

Product characteristics

STREAMLINE rProtein A is an affinity adsorbent for purifying monoclonal and polyclonal antibodies (62). It is based on highly cross-linked 4% agarose modified by including an inert metal alloy core to provide the required high density.

The ligand is a recombinant protein A specially engineered by fusing a cysteine residue to the C-terminus to favour an oriented coupling to the matrix by epoxy chemistry. This coupling generates a stable thioether linkage between the ligand and the STREAMLINE matrix. The oriented coupling provides high binding capacities for IgG due to the enhanced interaction between protein A and the F_c region of the antibody.

The recombinant protein A is produced in *E. coli* and purified by a multi-step chromatographic procedure before being coupled to the base matrix (59). The purification of protein A does not involve the use of coupled IgG or any other protein. The purified recombinant protein A is tested according to established specifications before being released for the manufacture of STREAMLINE rProtein A.

More information about STREAMLINE rProtein A, including instructions for use, is available in Data File STREAMLINE rProtein A (Code No. 18-1115-67), and in the Instructions included in the pack.

Table 16 summarizes the characteristics of STREAMLINE rProtein A.

Table 16. Characteristics of STREAMLINE rProtein A.

Ligand	Recombinant protein A *(E. coli)*
Ligand density	~6 mg protein A/ml gel
Particle size	80–165 µm
Approx. mean particle density	1.3 g/ml
Degree of expansion (H/H0) at 300 cm/h	2–3
pH stability[1]	
long term	3–10
short term	2–11
Binding capacity	
Total	~ 50 mg human IgG/ml gel
Dynamic[2]	~ 20 mg human IgG/ml gel
Storage	20% ethanol

Further information is available in Data File STREAMLINE rProtein A (Code No. 18-1115-67).

[1] Long term refers to the pH interval where the gel is stable over a long period of time without adverse effects on its subsequent chromatographic performance. Short term refers to the pH interval for regeneration and cleaning procedures.

[2] Breakthrough capacity, in packed and expanded bed, at a flow velocity of 300 cm/h. Sedimented bed height 15 cm.

Cleaning-in-place and sanitization-in-place

The pH stability of STREAMLINE rProtein A is limited by the susceptibility to hydrolysis of the protein A ligand at high pH values. This excludes using high concentrations of NaOH for cleaning and sanitization.

The following alternative cleaning-in-place protocol has therefore been developed for STREAMLINE rProtein A. It has been verified to restore both hydrodynamic and chromatographic performance over several purification cycles (46):

- 2 M NaCl and 1 mM NaOH in 20% ethanol, flow rate 100 cm/h, contact time 120 minutes
- 5% Sarcosyl[1], 20 mM EDTA, 0.1 M NaCl in 20 mM sodium phosphate, pH 7.0, 100 cm/h, contact time 90 minutes[2]
- 50 mM acetic acid in 20% ethanol (to remove the detergent), 300 cm/h, contact time 20 minutes

Other cleaning agents that can be recommended to remove strongly adsorbed, precipitated or denatured proteins are 6 M guanidine hydrochloride, 6 M urea and 1 M acetic acid.

Chapter 4 "Method Optimization" contains general information about cleaning-in-place of STREAMLINE adsorbents.

The following alternative protocols can be recommended to sanitize STREAMLINE rProtein A:

Protocol 1

Equilibrate the bed with a solution consisting of 2% hibitane digluconate and 20% ethanol. Allow to stand for 6 hours, then wash with at least 5 bed volumes of sterile binding buffer.

Protocol 2

Equilibrate the bed with a solution consisting of 0.1 M acetic acid and 20% ethanol. Allow to stand for 1 hour, then wash with at least 5 bed volumes of sterile binding buffer.

Protocol 3

Equilibrate the bed with 70% ethanol. Allow to stand for 12 hours, then wash with at least 5 bed volumes of sterile binding buffer. Note that specific regulations for classified areas and explosion-proof equipment may apply when handling large volumes of organic solvents.

Storage

We recommend storing STREAMLINE rProtein A in 20% ethanol.

Product availability

STREAMLINE rProtein A is supplied as a suspension in 20% ethanol in packs of 75 ml, 300 ml and 5 litres.

---

[1] Sodium N-lauroylsarcosinate (weak anionic detergent)

[2] The bed is equilibrated with 3 sedimented bed volumes and then left standing for 90 minutes.

STREAMLINE columns

Pharmacia Biotech manufactures a range of STREAMLINE columns specifically for use in expanded bed adsorption. STREAMLINE columns are optimized to give stable expanded beds with STREAMLINE adsorbents from small scale optimization, through pilot scale, and up to final production. Table 17 summarizes the STREAMLINE column range. More detailed information is available in the User Manual supplied with each column.

Table 17. STREAMLINE columns available from Pharmacia Biotech.

Column	Application area
STREAMLINE 25	Optimization work at laboratory scale
STREAMLINE 50	Optimization and verification work at pilot scale
STREAMLINE 200	Verification work at pilot scale and small scale production
STREAMLINE CD	Custom designed columns for full scale production

Column design

The unique design of the liquid distributor at the base of STREAMLINE columns ensures the formation of stable expanded beds. The distributor has been optimized for each column size to provide stable fluidization with STREAMLINE adsorbents at flow velocities between 200 and 500 cm/h, using water-based buffers as mobile phase.

STREAMLINE columns are also equipped with a movable adaptor operated by a hydraulic drive. This allows the height of the expanded bed to be altered during the different stages of an expanded bed adsorption cycle. The adaptor is lowered by pumping liquid into the hydraulic compartment above the adaptor plate. It is raised by pumping liquid upwards through the column while allowing the hydraulic liquid to flush out of the hydraulic compartment.

The adaptor is kept at its upper position for bed expansion and feed application and is lowered to the surface of the sedimented bed for desorption in packed bed mode. It can also be lowered to the surface of the expanded bed for the wash cycle after feed application. This speeds up the wash cycle and considerably reduces the consumption of wash buffer.

Large scale STREAMLINE CD columns, which are manufactured from stainless steel[1], can be supplied with an adsorbent sensor. The adsorbent sensor is mounted under the screen on the adaptor (see Fig. 29) and consists of a transmitter and a receiver. The transmitter sends out an ultrasonic signal and the receiver is adjusted to detect the signal in buffer solutions and fermentation broths containing cells etc., but not in solutions where adsorbent particles are present. The adsorbent sensor is

[1] STREAMLINE CD columns can also be delivered with a transparent column tube manufactured from cast polymethylmethacrylate (PMMA).

used to control the position of the adaptor during the run. It can also be used to back-flush the column if it detects build up of adsorbent particles under the adaptor net during feed-stock application. STREAMLINE CD columns are also supplied with an adaptor position indicator which provides continuous information about the position of the adaptor during the entire process.

STREAMLINE columns comply with hygienic requirements of process development and production, i.e. from laboratory to manufacturing scales. The hygienic design of small scale columns, such as STREAMLINE 25, as well as large scale STREAMLINE CD columns has been verified by sanitization studies. The columns were challenged with culture broths of yeast and bacteria to mimic a real expanded bed adsorption process (39, 52). Following a predefined sanitization-in-place protocol, the adsorbent, the hydraulic liquid, the column eluate and a number of critical test points in the column were all free from vegetative organisms.

STREAMLINE columns with inner diameters of 1000, 25 and 600 mm.

Table 18 lists the technical and operating characteristics of STREAMLINE columns. For more information about STREAMLINE CD columns, contact your nearest Pharmacia Biotech office.

Table 18. Technical and operating characteristics of STREAMLINE columns for expanded bed adsorption.

Property	STREAMLINE 25	STREAMLINE 50	STREAMLINE 200
Inner diameter	25 mm	50 mm	200 mm
Tube height	1000 mm	1000 mm	950 mm
Max. height[1] during operation	2300 mm	2300 mm	2400 mm
Foot-print of column stand	400 mm x 400 mm	300 mm x 300 mm	600 mm x 600 mm
Sedimented bed height			
min	10 cm	10 cm	10 cm
max	30 cm	30 cm	30 cm
Sedimented adsorbent volume			
min	0.05 L	0.2 L	3 L
max	0.15 L	0.6 L	9 L
Max. operating pressure	100 kPa (1 bar)	100 kPa (1 bar)	100 kPa (1 bar)
Recommended flow rates			
expanded mode; min	1 L/h (200 cm/h)	4 L/h (200 cm/h)	63 L/h (200 cm/h)
expanded mode; max	2.5 L/h (500 cm/h)	10 L/h (500 cm/h)	157 L/h (500 cm/h)
sedimented mode; elution	0.25-0.75 L/h (50-150 cm/h)	1-3 L/h (50-150 cm/h)	16-47 L/h (50-150 cm/h)

[1] Column with stand and adaptor in uppermost position.

Column materials

The materials used in STREAMLINE columns are compatible with chemicals and solvents commonly used to recover proteins from crude feedstock, and for cleaning and sanitization. Table 19 lists the principle components, together with the materials of construction.

Materials used in the construction of large scale STREAMLINE CD columns include electropolished stainless steel ASTM 316L (column tube[2], distributor plate, net, piping, fittings and valves), PTFE (valves), PP (adsorbent sensor) and EPDM (gaskets and seals).

Table 20 lists chemicals and solvents that can be used with STREAMLINE columns.

[2] STREAMLINE CD columns can also be delivered with a transparent column tube manufactured from cast polymethylmethacrylate (PMMA).

Table 19. Materials of construction.

Designation	STREAMLINE 25	STREAMLINE 50	STREAMLINE 200
Lid	PEEK[3]	PP[4]	SS[2] (ASTM 316L)
Column tube	Borosilicate glass	Borosilicate glass	Borosilicate glass
Top-piece	SS[2] (ASTM 316)	PP[4]	PP[4]
End-piece	PEEK[3]	PP[4]	SS[2] (ASTM 316L)
Net, top and bottom	PP[4] SS[1](ASTM 316)	SS[1] (ASTM 316L)	SS[1] (ASTM 316L)
Adaptor distributor plate	SS[2] (ASTM 316)	SS[2] (ASTM 316)	SS[2] (ASTM 316)
Bottom distributor plate	SS[2] (ASTM 316)	SS[2] (ASTM 316)	PVDF[5]
Adaptor	PTFE[6], PEEK[3] and SS[2] (ASTM 316)	PP[4], SS[2] (ASTM 316L)	SS[2] (ASTM 316L)
O-rings and gaskets	EPDM[7]	EPDM[7], NBR[8]	EPDM[7]

[1] Stainless steel

[2] Stainless steel, electropolished

[3] Polyetheretherketone

[4] Polypropylene

[5] Polyvinylidenefluoride

[6] Polytetrafluoroethylene

[7] Ethylenepropylenedimonomer

[8] Nitrile rubber (only in contact with hydraulic fluid)

Table 20. Chemicals and solvents that can be used with STREAMLINE columns.

Chemical agent	Concentration	Purpose	Comments
NaOH	1 M	CIP[1]/SIP[2]	Not recommended for STREAMLINE rProtein A and STREAMLINE Heparin
NaOH	0.01 M	Storage	Not recommended for STREAMLINE rProtein A and STREAMLINE Heparin
Ethanol	70%	CIP/SIP	
Ethanol	20%	Storage	
Isopropanol	30%	CIP	
NaCl	2 M	Regeneration/CIP	
HCl	0.01 M	CIP	Not recommended for STREAMLINE SP, STREAMLINE SP XL and STREAMLINE Heparin
Acetic acid	25%	CIP	Recommended for STREAMLINE SP and STREAMLINE SP XL only for a contact time of up to 30 minutes. Not recommended for STREAMLINE Heparin
Guanidine-HCl	6 M	CIP	
Urea	8 M	CIP	
Triton X-100	1%	CIP	
Tween	1%	CIP	
Glycerol	10–50%	Wash	

1 Cleaning-in-place

2 Sanitization-in-place

STREAMLINE systems

Manual systems

A portable system for operating STREAMLINE columns manually is available with two different tubing dimensions; 6 mm i.d. tubing for STREAMLINE 50 and 10 mm i.d. tubing for STREAMLINE 200. The system contains valves, tubing manifolds and an air trap, permitting complete operation of the column, including hydraulic control of adaptor movement and reverse flow. All components are assembled on a 700 x 550 mm stainless steel frame. The system is delivered with a stainless steel cover and handle, which makes it easy to relocate without disassembling.

The valves in the system are manual diaphragm valves with integrated tubing T-pieces (zero dead-leg valve), a design which reduces the system dead volume and eliminates stagnant areas where microbial growth could occur.

The system is supplied with four inlets for sample, buffers and CIP solution, and one extra outlet to collect product.

The connections in the system, including inlets, outlets and column connections, are all sanitary 25 mm o.d. clamp connections.

Manual STREAMLINE system

Pumps (one to pump liquid through the column and one for the hydraulic drive), monitors (UV, pH or conductivity), and tubings are not included and must be ordered separately. Extra tubing is needed to connect tanks to the system inlet and outlet valves, to connect the column (inlet, outlet and hydraulic inlet), to connect monitors between the flow reversal valve and the system outlet valve, to connect the pumps (inlet and outlet), and to connect the hydraulic liquid container to the system (to dispose of hydraulic liquid when lifting the adaptor). See Table 7 to select suitable pumps, tubings, monitors, etc.

Materials of construction are polypropylene (tubing manifolds, valves), EPDM (valves, gaskets, air trap), stainless steel ASTM 316L (air trap, 4-port 2-way valve), PTFE (4-port 2-way valve), and glass (air trap).

Pilot scale automated systems

Automated STREAMLINE systems are available in two sizes suitable for method development and small scale production. They have tubing sizes of 3 mm and 10 mm i.d. for STREAMLINE 50 and STREAMLINE 200 columns respectively. The larger tubing size can also accommodate custom designed columns with flow rates of around 400 litre/h. Table 21 lists technical data for the pilot scale automated STREAMLINE systems.

STREAMLINE pilot scale system for method development and small scale production.

Table 21. Technical data for STREAMLINE pilot scale systems.

	3 mm system	**10 mm system**
Tubing diameter	3 mm	10 mm
Operating pressure	2 bar	2 bar
Flow rate max.	12 L/h	180 L/h
Flow rate min.	0.6 L/h	9 L/h
Operating temperature	4–40 °C	4–40 °C

STREAMLINE pilot scale systems are equipped with the same valves, tubing and instruments used in BioProcess Systems supplied by Pharmacia Biotech for packed bed chromatography. The valve is a pneumatically driven diaphragm valve with an integrated tubing T-piece (zero dead-leg valve). This design reduces the length of the flow path and minimizes the dead volume of the system, thereby improving overall system performance and eliminating stagnant areas where microbial growth could occur. Sanitary TC connections are used throughout the system, including inlet, outlet and column connections. The hygienic status of this system has been verified in microbial challenge tests.

Monitors for recording UV, pH, conductivity, pressure and flow rate are included. Extra monitors may be added to the system for recording these parameters at additional locations in the liquid flow. A three-channel line recorder is available as an option for recording UV, pH and conductivity. All system pumps are peristaltic. Table 22 lists materials of construction.

Table 22. Materials of construction for STREAMLINE pilot scale systems.

Component	Material of construction
Tubing	PP[1]
Valves	PP[1], EPDM[2]
Air trap	Glass, Stainless steel (ASTM 316L), EPDM[2]
Pressure sensor	Stainless steel (ASTM 316L)
UV flow cell	Stainless steel (ASTM 316L), quartz
Conductivity flow cell and sensor	Stainless steel (ASTM 316L), PEEK[3]
pH flow cell and sensor	PP[1], glass
Flow monitor cell	Stainless steel (ASTM 316L), PFA[4]

[1] Polypropylene

[2] Ethylenepropylenedimonomer

[3] Polyetheretherketone

[4] Polyperfluoro alkoxy ethylene (Perfluoro (alkoxy alkane))

Production scale automated systems

Large scale STREAMLINE systems are custom designed to match the requirements of large scale STREAMLINE CD columns. These systems are based on the same design concept as the modular STREAMLINE systems used in process development and small scale production, which facilitates scale up. The main materials of construction are high alloy stainless steel, polypropylene, EPDM and glass. Sanitary TC connections are used throughout the system, including inlet, outlet and column connections.

The hygienic design of STREAMLINE production scale systems has been verified by sanitization studies. A system was challenged with culture broths of yeast and bacteria to mimic a real expanded bed adsorption process (39). Following a predefined sanitization-in-place protocol, a number of critical test points in the system were all free from vegetative organisms.

Fig. 29 shows the configuration of a STREAMLINE production scale system designed for the operation of a STREAMLINE 600 column (custom designed column with an internal diameter of 600 mm). For more information about custom designed STREAMLINE systems, contact your nearest Pharmacia Biotech office.

A production scale STREAMLINE system and column.

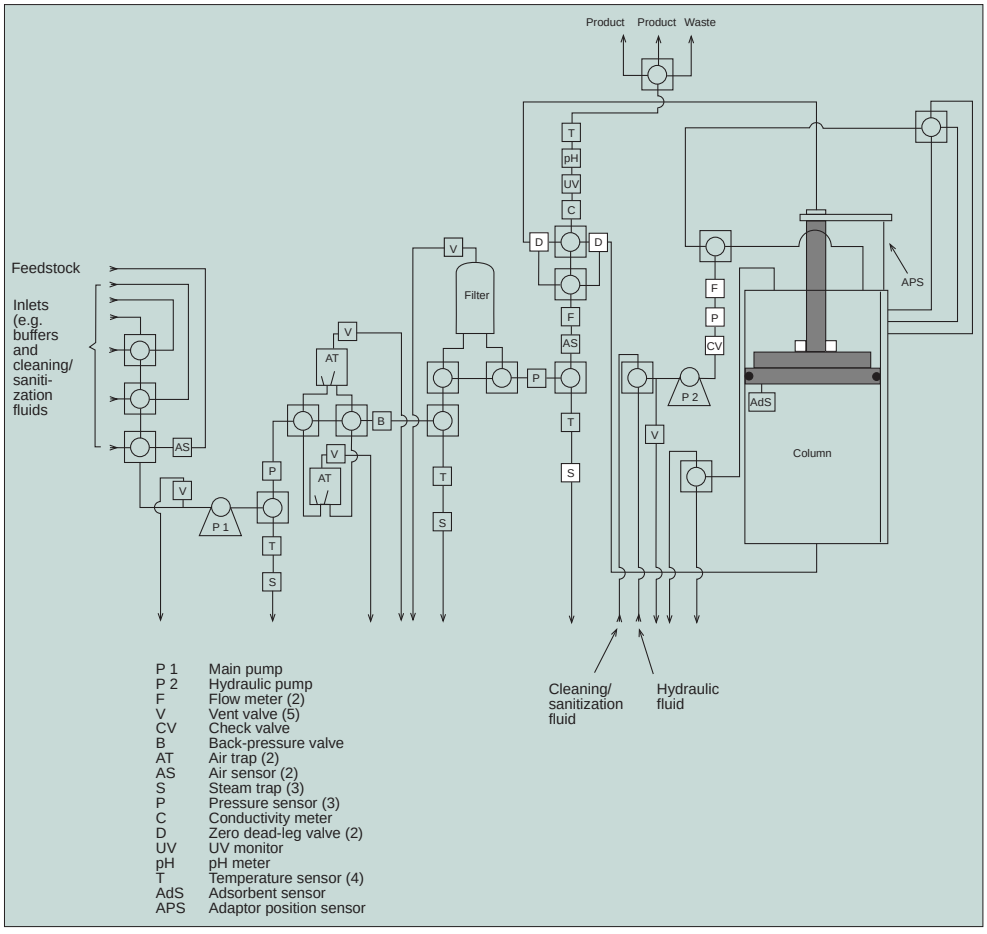


Fig. 29. STREAMLINE column and system for production scale operation.

System control

STREAMLINE pilot scale systems can be controlled by the Process Controller C-3 or by the UNICORN multipurpose controller. STREAMLINE production scale systems are controlled by UNICORN, or other commercially available control systems.

UNICORN adds flexibility. It enables more monitors to be connected and has a number of convenient programming features for method development and production. These, together with the intuitive user interface, simplify production.

UNICORN also provides data integrity, definable user access-levels and extensive batch documentation, which are important aspects of operating in GLP and GMP environments.

A UNICORN controlled system can be manually operated or fully automated through a user-defined programming sequence. For instance, the system can be programmed to respond with lifting or lowering the adaptor, flow stop, flow reversal (backflushing), lowered flow rate, etc. when the adsorbent sensor (STREAMLINE CD columns) detects adsorbent particles under the adaptor net.

Each STREAMLINE system houses UNICORN hardware. Up to four chromatographic systems can be connected to one PC running UNICORN control system software. For more information about UNICORN, contact your nearest Pharmacia Biotech office.

STREAMLINE production scale systems can also be supplied with a Programmable Logical Controller (PLC) as an alternative to UNICORN.

7. Applications

This Chapter shows how Expanded Bed Adsorption has been used successfully to capture target molecules from crude unclarified feed material. The applications cover different types of feed material and illustrate a variety of separation techniques, including ion exchange, affinity, and hydrophobic interaction chromatography.

Expanded Bed Adsorption in Capture from Bacterial Fermentation Cultures

This section describes adsorption from bacterial fermentation cultures. It includes applications where the target molecule is accumulated intracellularly in soluble form or as inclusion bodies, as well as where it is secreted into the cell culture broth.

Recovery of recombinant Annexin V from unclarified *E. coli* homogenate by expanded bed anion exchange adsorption

Expanded bed anion exchange adsorption has been used for pilot scale recovery of recombinant human placental annexin V from an Escherichia coli homogenate (28).

Annexin V is an anticoagulant protein found in placenta. Its molecular weight is approximately 34 kD and the isoelectric point is 4.9. It was cloned to be expressed intracellularly in *E. coli* and was released from the harvested cells by three passages at 700–900 bar through a high-pressure homogenizer. This procedure effectively disrupted the cells and also reduced the viscosity caused by released nucleic acids. Due to the tendency of the annexins to associate with membranous structures (phospholipids), a detergent (Triton X-100, 1% (v/v) final concentration) was added to the homogenate prior to purification. The biomass dry weight of the homogenate was 3.6%.

Method scouting was performed using clarified feed material on STREAMLINE DEAE packed in an XK 16 column to a bed height of 10 cm. When optimal conditions for feed conditioning, adsorption, washing, and elution had been defined, the method was optimized on a small expanded bed of STREAMLINE DEAE using crude, unclarified feed material to define optimal running conditions. The column used during optimization was a STREAMLINE 50 column with an internal diameter of 50 mm. The sedimented bed height was 15 cm. The method was finally scaled up to pilot scale in a STREAMLINE 200 column with an internal diameter of 200 mm.

Bed expansion/equilibration, feed application, and wash were performed at an upward flow velocity of 300 cm/h. The buffer used during expansion/equilibration and wash was 30 mM ammonium acetate, pH 5.5. Elution was performed at 100 cm/h using downward flow in sedimented bed mode. The elution buffer was 30 mM ammonium acetate containing 250 mM NaCl, pH 5.5.

Cleaning-in-place was performed after each purification cycle using upward flow with the adaptor positioned at twice the sedimented bed height. The cleaning protocol was: 0.5 M NaOH containing 1 M NaCl at a low flow velocity giving a contact time of at least 4 hours; 3 sedimented bed volumes of distilled water at 100 cm/h; 3 sedimented bed volumes of 30% isopropanol at 100 cm/h; 3 sedimented bed volumes of 25% acetic acid at 100 cm/h; and finally, adsorption buffer until the pH and conductivity of the outlet stream were the same as the buffer.

Figure 30 and Table 23 summarize the experiments. The yield of annexin V was approximately 95% as determined by an anticoagulant activity assay and reversed phase HPLC. No annexin V was found in the flow through or wash. Scanning SDS PAGE gels showed an increase in the annexin V content from 9% in the clarified homogenate to 20% in the eluate.

Table 23. Summary of results from lab scale and pilot scale expanded bed adsorption of recombinant annexin V from an unclarified *E. coli* homogenate.

	STREAMLINE 50	**STREAMLINE 200**
Volumes (L)		
Fermentation	3.4	50
Homogenate	1.7	26.5
Adsorbent	0.3	4.7
Wash	4.5 (15 bed vol)	71 (15 bed vol)
Eluate	0.6 (2 bed vol)	10 (2.1 bed vol)
Time (min)		
Column set-up, bed expansion, and equilibration	60	60
Feed-stock application	17	17
Wash	46	45
Elution	18	19
TOTAL	2 h 21 min	2 h 21 min
Yield	> 95%	> 95%

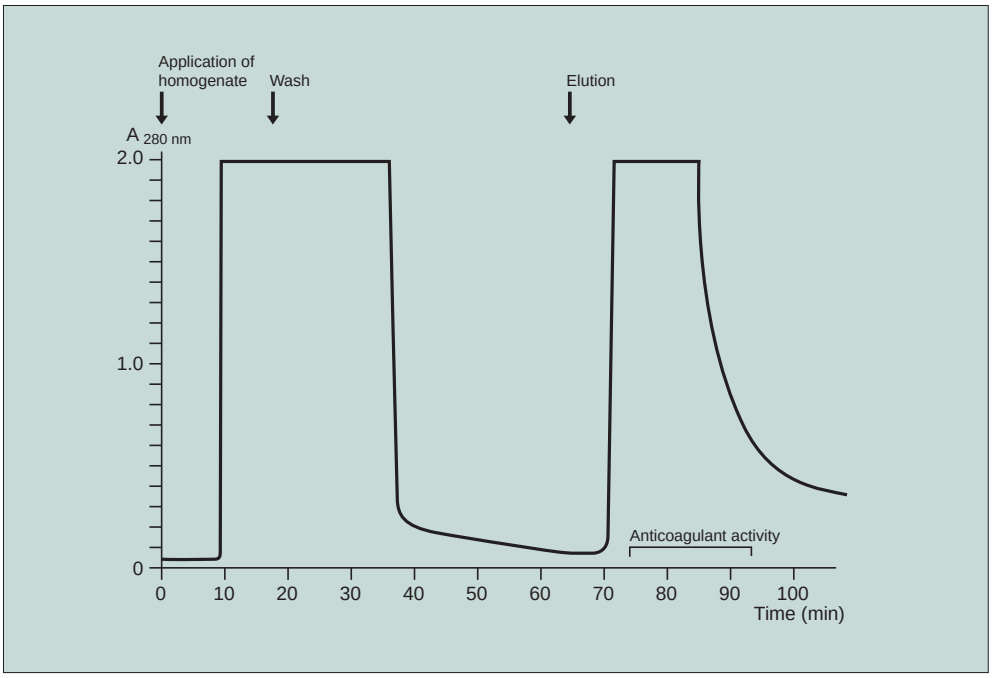


Fig. 30. Chromatogram from pilot scale expanded bed adsorption of recombinant annexin V from unclarified E. coli homogenate using a STREAMLINE 200 column with STREAMLINE DEAE adsorbent. (Work from Pharmacia Biotech, Uppsala, Sweden).

Purification of a therapeutic recombinant protein from unclarified *E. coli* homogenate by expanded bed cation exchange adsorption

The use of expanded bed cation exchange adsorption to purify a recombinant protein from *Escherichia coli* homogenate has been reported by Rhône Poulenc Rorer GENCELL, France (58).

The protein, which had a molecular weight of 17 kD, was expressed intracellularly in soluble form in *E. coli* and was released from the harvested cells by high pressure homogenization at 1000 bar. Expanded bed adsorption on STREAMLINE SP was the first step (capture step) in a sequence of three chromatographic steps to purify this protein, which was intended to be used in clinical trials.

Method scouting was performed using clarified feed material on STREAMLINE SP packed in an XK 26 column to a bed height of 5 cm. After having defined optimal running conditions, unclarified feed-stock was used for optimization in expanded mode on a STREAMLINE 50 column (50 mm i.d.) containing 400 ml of adsorbent, providing a sedimented bed height of 20 cm. The method was finally scaled up to pilot scale in a STREAMLINE 200 column (200 mm i.d.) containing 8 litres of adsorbent. Seventy-six litres of unclarified feed material containing 410 g of protein were applied to the STREAMLINE 200 column.

Cleaning-in-place was performed after each purification cycle by exposing the adsorbent to 0.5 M NaOH for one hour without flow. This solution was then replaced by 0.1 M NaOH or 20% ethanol for storage until the next run. More than 80 capture cycles, followed by CIP, were successfully run on the same STREAMLINE SP adsorbent. Recovery varied between 73% and 156% as measured by reversed phase HPLC (Fig. 31). Recovery for the same *E. coli* strain and plasmid was 85 ±22% (n=6).

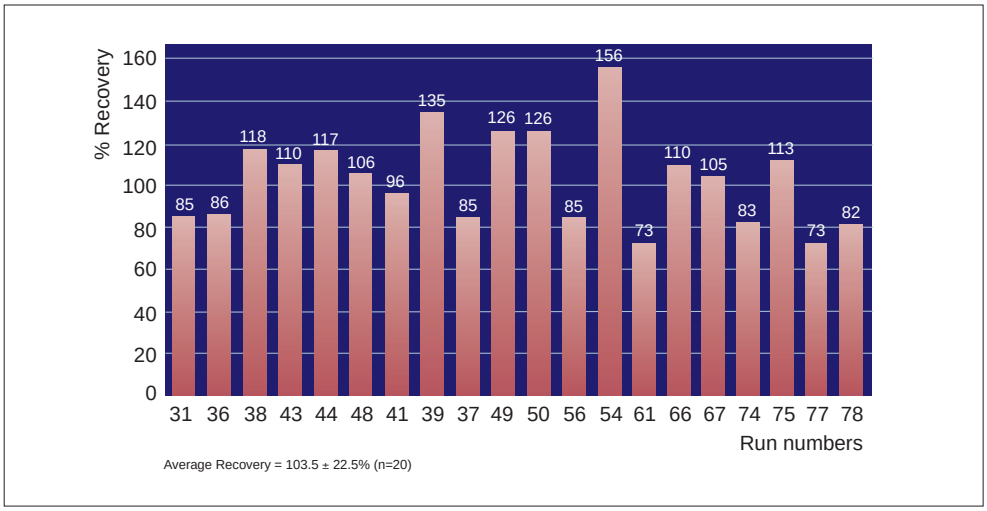


Fig. 31. Reproducibility study of recovery from a STREAMLINE SP used in STREAMLINE 50 column. Eighty runs on the same column and adsorbent were performed with two *E. coli* strains and four gene constructs. Recovery was measured by RP-HPLC (Reproduced with permission, from ref. 58).

The expanded bed adsorption step on STREAMLINE SP reduced total protein content 10-fold and resulted in a 3-fold increase in concentration of the target protein. The purity was consistent between the two scales as determined by reversed phase HPLC. Table 24 summarizes the efficiency of reduction of total protein, endotoxin and DNA over the STREAMLINE SP step. Since the endotoxin levels were low and consistent from batch to batch, it was concluded that CIP with 0.5 M NaOH efficiently removed endotoxin from the column.

Table 24. Removal of total protein, endotoxins and contaminating DNA during the expanded bed adsorption step on a STREAMLINE 50 column. Protein concentration was determined by BCA[1], endotoxin activity with the Limulus Amebocyte Lysate (LAL) kit (Bio-Whittaker), and DNA with the Threshold system (Molecular Devices Inc.).

	Total protein mg	Specific Activity[2]	Endotoxins EU/ml	DNA ng/ml
Starting material	19 000	0.1	1 250 000	1 300 000
STREAMLINE SP eluate	2 317	0.65	1 250	15

[1] Bicinchoninic acid (trademark owned by Pierce)

[2] Specific activity represents the amount of therapeutic protein (in mg) per mg of total protein.

Purification of a recombinant anti-HIV Fab-fragment from *E. coli* homogenate by expanded bed cation exchange adsorption

Expanded bed cation exchange adsorption on STREAMLINE SP was used in the capture step during purification of a recombinant anti-HIV Fab-fragment from an *Escherichia coli* homogenate (29).

The work was performed by Pharmacia Biotech in collaboration with the Karolinska Hospital, Stockholm, Sweden, and the Swedish Institute for Infectious Disease Control, Stockholm, Sweden, with the purpose of producing Fab-fragments active in neutralizing human immunodeficiency virus type 1 (HIV-1). The Fab-fragment was directed against the envelope protein gp120 of the HIV-1 virus. The Fab-fragment was expressed in the periplasm of *E. coli* and was released from the harvested cells by 3 passages through a high pressure homogenizer at a pressure of 700-800 bar. An endonuclease (Benzonase, Merck, Nycomed Pharma A/S) was added to the buffer used during homogenization at a ratio of 10 µl per 4 litre of buffer to reduce the viscosity. The biomass dry weight of the homogenate was 1.4%. The isolelectric point of the Fab-fragment was determined to be 10.3.

Method scouting was performed on a small packed bed of STREAMLINE SP using clarified feed material. Method optimization in expanded mode using crude, unclarified cell homogenate was performed with a STREAMLINE 50 column (50 mm i.d.) containing 300 ml adsorbent corresponding to a sedimented bed height of 15 cm. The method was finally scaled up to pilot scale in a STREAMLINE 200 column (200 mm i.d.). containing 4.7 litres of adsorbent. The flow velocity during expansion/equilibration, adsorption and wash was 300 cm/h, which caused the bed to expand 2.8 times during expansion/equilibration and 3.4 times during feed application. The buffer used during expansion/equilibration and wash was 50 mM sodium acetate, pH 5.0. Desorption of the Fab-fragment from the adsorbent was performed with downward flow in sedimented mode using 50 mM sodium acetate, pH 5.0 containing 1 M NaCl. The flow velocity during desorption was 100 cm/h.
Fig. 32 shows a chromatogram from the run on the STREAMLINE 50 column. Table 25 summarizes the experiments at the two scales. The same fermentation batch was used for both runs but homogenization was performed at different times.

Table 25. Summary of results from lab scale and pilot scale expanded bed adsorption of recombinant anti-HIV Fab-fragment from unclarified *E. coli* homogenate.

	STREAMLINE 50	**STREAMLINE 200**
Volumes (L)		
Adsorbent	0.3	4.7
Homogenate	4.85	60
Eluate	0.50	6.0
Fab-conc. (µg/ml)	6.8 (in) 62.5 (out)	23 (in) 122.5 (out)
Yield (%)	95	100

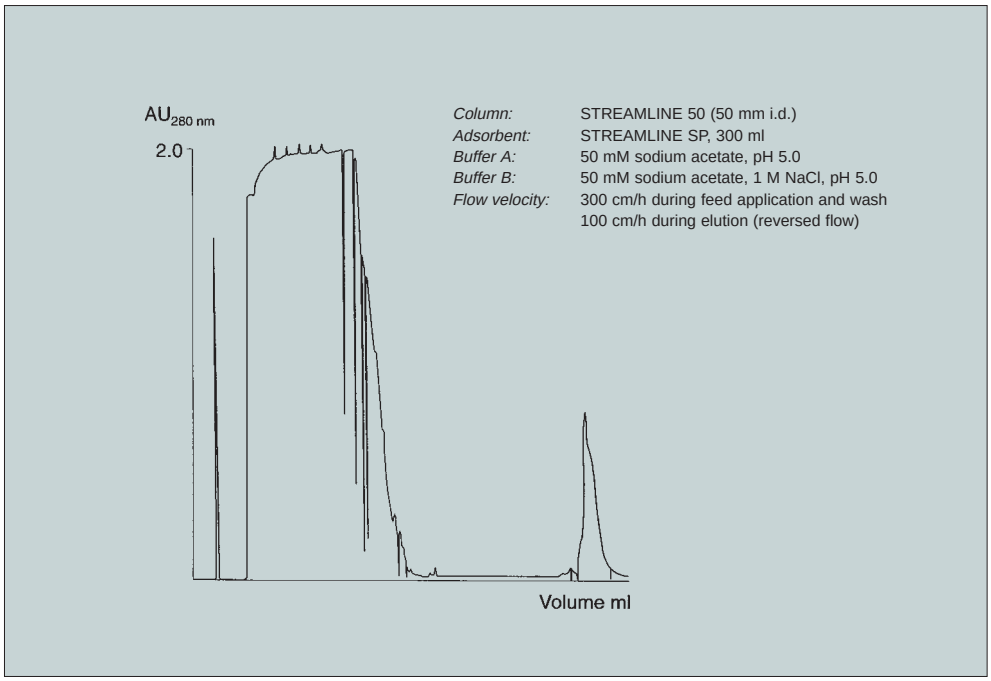

AU280 nm
2.0
Column: STREAMLINE 50 (50 mm i.d.)
Adsorbent: STREAMLINE SP, 300 ml
Buffer A: 50 mM sodium acetate, pH 5.0
Buffer B: 50 mM sodium acetate, 1 M NaCl, pH 5.0
Flow velocity: 300 cm/h during feed application and wash
100 cm/h during elution (reversed flow)
Volume ml

Fig. 32. Capture of recombinant anti-HIV Fab-fragment on STREAMLINE SP. (Work by Pharmacia Biotech, Uppsala, Sweden).

Following Capture on STREAMLINE SP, the Fab-fragment was further purified by Intermediate Purification on Phenyl Sepharose 6 Fast Flow (high sub) and by a Polishing step on SOURCE 15S. This three-step procedure, starting with crude, unclarified homogenate, resulted in a pure Fab-fragment according to SDS-PAGE. It was also found to have retained immunological activity towards the surface protein gp120 of HIV-1, as determined by ELISA, and retained neutralizing activity towards HIV-1. The neutralizing activity was measured as the Fab-fragment's ability to inhibit the HIV-1 infection of T-cells in *in vitro* cell cultures. Table 26 summarizes the complete purification process.

Table 26. Summary of results from the three-step procedure for the purification of recombinant anti-HIV Fab-fragment.

Purification step	Volume (litres)	Fab-conc. (µg/ml)	Step recovery (%)
Bacterial homogenate	4.85	6.8	
STREAMLINE SP	0.5	62.5	95
Phenyl Sepharose 6 Fast Flow (high sub)	0.026	122.5	96
SOURCE 15S	0.005	940	56

After each purification cycle on the STREAMLINE SP adsorbent, the column was subjected to a cleaning-in-place procedure using upward flow with the adaptor positioned at twice the sedimented bed height. The cleaning protocol consisted of: 0.5 M NaOH containing 1 M NaCl at a flow velocity of 30 cm/h giving a contact time of 4 hours; 3 sedimented bed volumes of distilled water (room temperature) at 100 cm/h; 10 sedimented bed volumes of distilled water (85-95 °C) at 100 cm/h; 1 sedimented bed volume of 25% acetic acid, 20% ethanol at 100 cm/h; and finally, 10 sedimented bed volumes of adsorption buffer at 100 cm/h.

To verify the function of the adsorbent after repeated use, the STREAMLINE 50 column was subjected to 50 subsequent purification cycles, each cycle followed by the CIP protocol described above. The feed material used in this study was prepared by releasing the Fab-fragment from the cells by osmotic shock (by sucrose) instead of high pressure homogenization. An endonuclease (Benzonase, Merck, Nycomed Pharma A/S) was added to the lysate to reduce viscosity. The degree of expansion was determined before each cycle. The number of theoretical plates of the expanded bed was determined before cycle 1 and after cycle 20. Breakthrough capacity for lysozyme was determined before cycle 1 and after cycles 30 and 50. The breakthrough capacity was determined in expanded bed mode. Table 27 summarizes the results.

Table 27. Summary of results from a study on the re-useability of STREAMLINE SP.

	Start	20 cycles	30 cycles	50 cycles
Degree of expansion (H/H_0)[1]	3.2	3.2	3.1	3.2
Number of theoretical plates	31	27	n.d.	n.d.
Breakthrough capacity for lysozyme (mg lysozyme/ml sedimented bed)	85	n.d.	85	85

[1] H = expanded bed height when the adsorbent has been expanded and equlibrated with start buffer

H_0 = sedimented bed height

The results indicate that the adsorbent could be reused for more than 50 cycles without compromising its function. No effect on the tested parameters could be seen over 50 cycles. SDS-PAGE on collected fractions revealed no loss in chromatographic performance over the 50 cycles.

Purification of a recombinant Pseudomonas aeruginosa exotoxin A from unclarified *E. coli* lysate by expanded bed anion exchange adsorption

Expanded bed anion exchange adsorption has been used in the purification of a genetically modified recombinant *Pseudomonas aeruginosa* exotoxin A expressed in the periplasm of *Escherichia coli* (53).

Gram quantities of inactivated *Pseudomonas aeruginosa* exotoxin A were needed to prepare several polysaccharide conjugated vaccines. The inactivated exotoxin A serves as the carrier protein moiety that is covalently bound to the polysaccharide moiety. The conjugate was to be used as a vaccine against certain pathogenic strains of methicillin-resistant *Staphylococcus aureus* and *Shigella*. The genetically modified, inactivated exotoxin A was cloned into *E. coli* and expressed as a soluble protein in the periplasm of the recombinant bacteria.

The harvested bacterial cells (4.5 kg) were suspended in 2 volumes of 20% sucrose in 50 mM Tris buffer, pH 7.4, containing 1 mM EDTA. The exotoxin A was released by osmotic schock after dilution with 18 volumes of 50 mM Tris buffer, pH 7.4. An endonuclease (Benzonase, Merck, Nycomed Pharma A/S) was added at a ratio of 0.2 µl (75 U) per gram cells and the suspension was diluted with 18 volumes of the Tris buffer. The endonuclease was needed to reduce the viscosity of the cell extract. The concentration of dry cell mass in the cell extract was 6 g/L.

The unclarified cell extract was applied to STREAMLINE DEAE adsorbent, expanded and equilibrated with 50 mM Tris buffer, pH 7.4, in a STREAMLINE 200 column (200 mm i.d.). The column contained 4.7 litres of adsorbent providing a sedimented bed height of 15 cm. The adsorbent expanded 5 times during the adsorption phase. After loading, the adsorbent was washed in expanded mode with 40 litres (8.5 sedimented bed volumes) of buffer. Elution was performed with downward flow in sedimented bed mode using a flow velocity of 100 cm/h. The buffer used during expansion/equilibration and wash was 50 mM Tris buffer, pH 7.4. The elution buffer was 50 mM Tris buffer, pH 7.4, containing 0.5 M sodium chloride.

Cleaning-in-place was performed after each purification cycle using upward flow with the adaptor positioned at twice the sedimented bed height. The cleaning protocol was: 0.5 M NaOH containing 1 M NaCl at a low flow rate, giving a contact time of at least 4 hours; 3 sedimented bed volumes of distilled water at 100 cm/h; 3 sedimented bed volumes of 30% isopropanol at 100 cm/h; 3 sedimented bed volumes of 25% acetic acid at 100 cm/h; and finally, adsorption buffer until the pH and conductivity of the outlet stream were the same as the buffer.

Fig. 33 shows the expanded bed adsorption step on STREAMLINE DEAE.

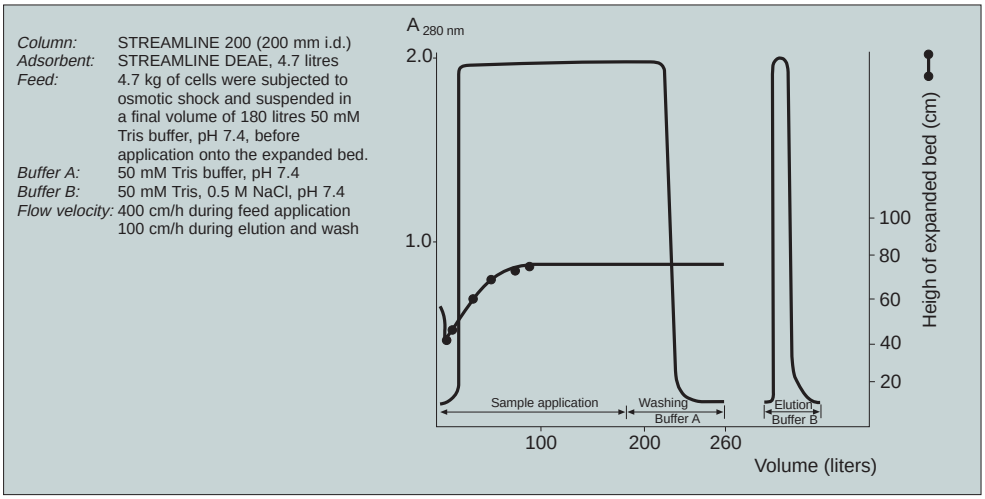

Column: STREAMLINE 200 (200 mm i.d.)
Adsorbent: STREAMLINE DEAE, 4.7 litres
Feed: 4.7 kg of cells were subjected to osmotic shock and suspended in a final volume of 180 litres 50 mM Tris buffer, pH 7.4, before application onto the expanded bed.
Buffer A: 50 mM Tris buffer, pH 7.4
Buffer B: 50 mM Tris, 0.5 M NaCl, pH 7.4
Flow velocity: 400 cm/h during feed application
100 cm/h during elution and wash
A 280 nm
2.0
1.0
Sample application
Washing
Buffer A
Elution
Buffer B
100
200
260
Volume (liters)
100
80
60
40
20
Heigh of expanded bed (cm)

Fig. 33. Capture of recombinant Pseudomonas aeruginosa exotoxin A on STREAMLINE DEAE. (Work by National Institute of Health, Bethesda, Maryland, USA, in collaboration with Pharmacia Biotech, Uppsala, Sweden).

Following the Capture step on STREAMLINE DEAE, the material was further purified by two Intermediate Purification steps and a final Polishing step. The first intermediate purification step was hydrophobic interaction chromatography (HIC) on Phenyl Sepharose 6 Fast Flow (high sub) packed in a BPG column (Pharmacia Biotech). This step removed a substantial part of the UV absorbing material that could interfere with the following steps. The second intermediate purification step was anion exchange chromatography on SOURCE 30Q packed in a FineLINE 100 column. This step removed the majority of the remaining contaminants. The polishing step was HIC on SOURCE 15 PHE. The four-step procedure, starting with crude unclarified lysate, resulted in a pure protein according to PAGE and RPC analysis, with an overall recovery of 51%. Table 28 summarizes the purification process.

Table 28. Purification table for the complete process. Values are extrapolated from actual runs, except for STREAMLINE DEAE, which was performed at the scale given in the table.

Purification step	Volume (litres)	Total protein (gram)	Exotoxin A (gram)[1]	Step recovery (%)
Bacterial extract	180	351.0	10.8	
STREAMLINE DEAE	13.5	140.0	8.54	79
Phenyl Sepharose Fast Flow (high sub)	11.4	41.0	6.60	77
SOURCE 30Q	30.2	12.6	6.04	91
SOURCE 15PHE	12.2	n.d.	5.5	91

[1] Activity was determined with a radial immunodiffusion assay using Goat anti-exotoxin A antibodies (List, USA).

The Capture step using expanded bed adsorption with crude, unclarified feed material was compared with traditional processing using clarified feed material on a packed bed of chromatography media. In the packed bed process, the cell lysate was centrifuged, clarified and applied on a packed bed of the anion exchanger DEAE Sepharose Fast Flow at a flow velocity of 100 cm/h. The protein was eluted with 8 bed volumes of a linear gradient from 0 to 0.5 M NaCl. In total, 73% of the initial exotoxin A was recovered with a specific activity of 0.1 mg toxin/mg protein. The total processing time for 4.5 kg cells is calculated to be 8-10 hours.

The protein was eluted from the expanded bed with a specific activity of 0.06 mg toxin /mg protein. The processing time was 2.5 hours. Thus, processing the crude, unclarified feed material directly on an expanded bed adsorption column was 3 times faster than traditional processing using clarified feed on a packed chromatography column. Although the specific activity of the exotoxin eluted from the expanded bed was lower, the yield was slightly higher.

Table 29 compares the capture step peformed on the expanded bed with that on the packed bed.

Table 29. Comparing capture steps for processing of 4.5 kg *E. coli* cells for the production of recombinant *Pseudomonas aeruginosa* exotoxin A.

	DEAE Sepharose Fast Flow[1]	STREAMLINE DEAE
Volumes (L)		
Cell lysate	90	180
Eluate	36	13.5
Time (hrs)		
Centrifugation	2–3	-
Clarification	2–3	-
Loading, wash, elution	3	2.5
Specific activity (mg toxin/mg protein)	0.1	0.06
Yield (%)	73	79

[1] Packed column numbers are extrapolated values from a run on an XK 16 column with a packed bed volume of 21 ml.

Recovery of recombinant human Interleukin 8 from *E. coli* inclusion bodies by expanded bed cation exchange adsorption

Expanded bed cation exchange adsorption has been used to recover human interleukin 8 (IL-8) expressed in *E. coli* as inclusion bodies (31).

Human IL-8, a pro-inflammatory cytokine with a molecular weight of 8.3 kD and an isoelectric point of 9, was expressed in *E. coli* both in a soluble form and as inclusion bodies. To disrupt the cells and solubilize IL-8, the harvested cells were resuspended in a solution containing 6 M guanidine hydrochloride in 30 mM sodium phosphate, pH 6.5. The amount of 6 M guanidine hydrochloride solution

added per gram cells (wet weight paste) was 2–3 ml. The suspension was stirred at room temperature for approximately 3 hours. IL-8 was then renatured by dilution with water in two steps. The first dilution was performed with 3 volumes of water followed by stirring at room temperature for 30 minutes. In the second step, it was again diluted with 3 volumes of water and stirred at room temperature overnight. The resulting unclarified debris and precipitate-containing feed-stock had a conductivity of 27 mS/cm. The pH was 6.6. Biomass content in the final feed-stock was approximately 1% dry weight.

The crude, unclarified feed-stock was applied to a STREAMLINE 50 column (50 mm i.d.) containing 300 ml STREAMLINE SP adsorbent corresponding to a sedimented bed height of 15 cm. The flow velocity during expansion/equilibration, adsorption and wash was 300 cm/h. The buffer used during expansion/equilibration and wash was 30 mM sodium phosphate, pH 6.5. Desorption of IL-8 from the adsorbent was performed with downward flow in sedimented mode using 30 mM sodium phosphate, pH 6.5, containing 0.5 M NaCl. The flow velocity during desorption was 100 cm/h.

Table 30 summarizes the adsorption step on STREAMLINE SP. An 11-fold concentration of IL-8 was achieved. The yield of IL-8 in the eluate, as determined by FEIA, was 97% and the purification factor was 4.8.

Table 30. Summary of results from expanded bed adsorption of recombinant IL-8 from unclarified *E. coli* feed-stock.

	Volume (ml)	Total protein (mg)	IL-8 (mg)	Yield (%)
Fermentation	6700	-	-	-
Feed-stock	12080 (40 s.b.v.)	3745	870	100
Flow-through and wash	13800 (46 s.b.v.)	2990	30	3
Eluate	1075 (3.6 s.b.v.)	750	840	97

[1] s.b.v. = sedimented bed volumes

After each purification cycle on the STREAMLINE SP adsorbent, the column was subjected to a cleaning-in-place procedure using upward flow with the adaptor positioned at twice the sedimented bed height. The cleaning protocol consisted of: 1% (w/v) DARACLEAN[1] 8471 at a flow velocity of 30 cm/h, giving a contact time of 4 hours, followed by 4 sedimented bed volumes of adsorption buffer at a flow velocity of 100 cm/h.

[1] DARACLEAN (Grace Dearborn Ltd) is a commercially available cleaning agent containing caustic soda, alkaline salts and the non-ionic detergent Triton CF 10.

To verify the function of the adsorbent after repeated use, the STREAMLINE 50 column was subjected to 50 subsequent purification cycles, each followed by the CIP protocol described above. The degree of expansion was determined before each cycle and breakthrough capacity for lysozyme was determined before cycle 1 and after cycles 25 and 50. Breakthrough capacity was determined in expanded bed mode. Table 31 summarizes the results.

Table 31. Summary of results from the study on the re-useability of STREAMLINE SP.

	Start	25 cycles	50 cycles
Degree of expansion (H/H_0)[1]	2.8	2.4	2.5
Breakthrough capacity for lysozyme (mg lysozyme/ml sedimented bed)	69	71	74

[1] H = expanded bed height when the adsorbent has been expanded and equlibrated with start buffer

H_0 = sedimented bed height

The results indicated that the adsorbent could be reused for more than 50 cycles without compromising its function. No effect on the tested parameters could be seen over 50 cycles. SDS-PAGE on collected fractions revealed no loss in chromatographic performance over the 50 cycles.

Purification of a secreted recombinant protein from *E. coli* fermentation broth by expanded bed anion exchange adsorption

Expanded bed anion exchange adsorption on STREAMLINE DEAE was used in the purification of a recombinant fusion protein directly from crude *Escherichia coli* fermentation broth (32).

The fusion protein consisted of two synthetic IgG-binding domains (ZZ) derived from staphylococcal protein A and a repeat-structure (M5) from the central repeat region of the malaria antigen Pfl55/RESA. An efficient and reproducible production/purification scheme is of interest since ZZ-M5 and related fusion proteins have been discussed as possible components in a blood-stage malaria vaccine.

The rational behind the design of the fusion protein was to achieve a low combined isoelectric point to enable selective recovery using a DEAE anionic adsorbent at relatively low pH. The isoelectric point of the fusion protein was 4.5, which allowed adsorption on the STREAMLINE DEAE adsorbent at pH 5.5, where most of the *E. coli* proteins are not adsorbed.

The fusion protein was secreted into the culture medium in high yields to give a final concentration of approximately 550 mg/L in the fermentation broth. The cell density in the fermentation broth was 10^{11} cfu/ml and the dry weight was 37 g/L.

The crude fermentation broth, adjusted to pH 5.5, was loaded directly onto a

STREAMLINE 50 column (50 mm i.d.) containing 200 ml STREAMLINE DEAE corresponding to a sedimented bed height of 10 cm. The conductivity and viscosity of the broth was adjusted by online 1:1 mixing with loading buffer immediately before the broth entered the column. Online dilution was applied to minimize cell lysis, since release of negatively charged DNA might reduce the capacity of the anion exchange adsorbent and significantly increase the viscosity of the fermentation broth. The flow velocity during expansion/equilibration, adsorption and wash was 200 cm/h. Desorption of the fusion protein was performed with downward flow in sedimented bed mode using 0.5 M NaCl. The flow velocity during desorption was 100 cm/h.

Following the initial purification step on STREAMLINE DEAE, the fusion protein was further purified by affinity chromatography on IgG Sepharose, utilizing the IgG affinity of the fusion partner ZZ. Table 32 summarizes the results from both purification steps. As determined by SDS-PAGE, the eluate from the expanded bed contained almost exclusively ZZ, indicating the low amounts of extracellular *E. coli* proteins that bind to an anion exchanger at pH 5.5. The second purification step on IgG Sepharose gave no further visual purification of ZZ-M5 but was included as a polishing step to reduce the amount of DNA and endotoxin in the final product. Considering an immunization dose of 50-100 µg protein, the levels of DNA and endotoxin after the two-step purification procedure were in the range acceptable by regulatory authorities (10-100 pg DNA/dose and 10 EU/mg product, respectively). The expanded bed adsorption also enabled efficient cell removal and a 16-fold volume reduction. It was concluded by viable count measurements that 99.99% of the cells could be removed by washing the expanded bed with six bed volumes. The whole expanded bed process was completed within four hours and the entire procedure, from inoculation of the fermentor to the recovery of the purified product, was completed within two working days.

Table 32. Summary of results from the two-step procedure for the purification of the recombinant fusion protein ZZ-M5.

Purification step	Volume (litres)	ZZ-M5 (gram)	DNA (pg/mg)	Endotoxin (EU/mg)	Step recovery (%)
Fermentation	8 (40 s.b.v.)	4.4	n.d.	n.d.	
STREAMLINE DEAE					
Wash	6 (30 s.b.v.)				
Eluate	0.5 (2.5 s.b.v.)	4.1	60 000	12 000	93
IgG Sepharose	0.5	4.1	700	10	100

[1] s.b.v. = sedimented bed volumes

Recovery of recombinant protein A from *E. coli* fermentation broth by expanded bed adsorption on the high capacity anion exchange adsorbent STREAMLINE Q XL

Expanded bed adsorption on STREAMLINE Q XL was used to recover a recombinant protein A directly from crude, unclarified *Escherichia coli* fermentation broth. (Work by Pharmacia Biotech, Uppsala, Sweden).

The recombinant protein A was expressed in *E. coli* at an expression level of 0.4 mg/ml cell culture suspension. It had a molecular weight of 34 kDa and the isolelectric point was 4.5. The cell culture suspension was diluted to a final conductivity of 5.3 mS/cm using one part distilled water and one part 10 mM Tris/HCl buffer, pH 7.4. Benzonase (Merck, Nycomed Pharma A/S) (5 µl/L) and $MgCl_2$ (11 mM) was added and the cell culture suspension was then allowed to stand under gentle stirring for approximately 60 minutes at room temperature before application to the expanded bed. (Benzonase is an endonuclease added to reduce viscosity by degrading released nucleic acids. Mg^{2+} neutralizes negative charges on nucleic acids present in the feed. This prevents the nucleic acids from binding to the cationic ligands of the adsorbent, which will otherwise reduce binding capacity for the target protein.)

Method scouting was performed in a packed bed of STREAMLINE Q XL using clarified feed material. Optimal conditions for binding, wash and elution were defined. The breakthrough capacity, as determined in the packed bed experiments, was found to be approximately 15 mg recombinant protein A per ml adsorbent.

Method optimization in expanded mode was performed on a STREAMLINE 25 column (25 mm i.d.). The column contained 75 ml of STREAMLINE Q XL, which corresponds to a sedimented bed height of 15 cm.

The method was finally scaled up to pilot scale in a STREAMLINE 200 column (200 mm i.d.).

Bed expansion/equilibration, feed application and wash were performed at an upward linear flow velocity of 400 cm/h. The buffer used during expansion/equilibration and wash was 10 mM Tris/HCl, pH 7.4, containing 10 mM $MgCl_2$. Elution was performed at 100 cm/h using downward flow in sedimented mode. The elution buffer was 10 mM Tris/HCl, pH 7.4, containing 1 M NaCl.
After elution, the bed was subjected to cleaning-in-place by washing with a solution of 0.5 M NaOH and 1 M NaCl. The cleaning solution was applied with upward flow with the adaptor positioned at twice the sedimented bed height.

Figure 34 shows a chromatogram from a laboratory scale run in a STREAMLINE 25 column. Table 33 summarizes the experiments at both laboratory and pilot scale. The difference in yield between the different scales is attributed to variation during assay of protein A concentration in the crude feed-stock.

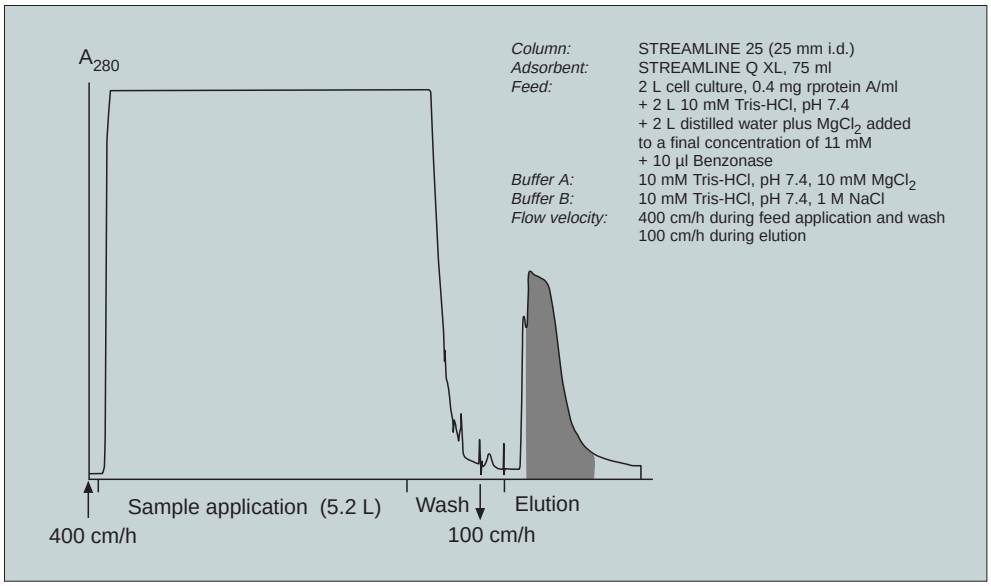

A280
Column: STREAMLINE 25 (25 mm i.d.)
Adsorbent: STREAMLINE Q XL, 75 ml
Feed: 2 L cell culture, 0.4 mg rprotein A/ml
+ 2 L 10 mM Tris-HCl, pH 7.4
+ 2 L distilled water plus MgCl2 added
to a final concentration of 11 mM
+ 10 µl Benzonase
Buffer A: 10 mM Tris-HCl, pH 7.4, 10 mM MgCl2
Buffer B: 10 mM Tris-HCl, pH 7.4, 1 M NaCl
Flow velocity: 400 cm/h during feed application and wash
100 cm/h during elution
Sample application (5.2 L)
Wash
Elution
400 cm/h
100 cm/h

Fig. 34. Chromatogram from laboratory scale expanded bed adsorption of recombinant protein A from unclarified *E. coli* cell culture suspension using STREAMLINE Q XL in a STREAMLINE 25 column. (Work by Pharmacia Biotech, Uppsala, Sweden).

Table 33. Summary of results from expanded bed adsorption of recombinant protein A at laboratory and pilot scale.

	STREAMLINE 25	STREAMLINE 200
Fermentor size (L)	10	100
Sample dilution	3x	3x
Feed volume (L)	5.2	300
Yield (%)	107	80
Total protein reduction	2.9	2.6
DNA reduction	12	13

Purification of human recombinant Interleukin 1 Receptor Antagonist from *B. subtilis* fermentation broth by expanded bed cation exchange adsorption

Human interleukin 1 receptor antagonist (IL-1ra) and IL-ra mutants, expressed in *Bacillus subtilis*, have been purified by expanded bed adsorption on STREAMLINE SP (72).

IL-1ra is the antagonist member of the interleukin 1 (IL-1) family. It exerts its activity by competing with IL-1 for binding to its receptors. The inhibitory activity of IL-1ra on the effect of IL-1 makes it a candidate for therapeutic use in a number of pathologies in which IL-1 activity is involved.

IL-1ra was expressed in *B. subtilis* in endocellular form and released into the culture medium by starvation-induced sporulation, a process which allows spontaneous release of the recombinant protein without need for a specific cell disruption step. The sporulation was induced within the fermentor by simultaneous starvation and temperature shift.

IL-1ra was purified from the culture medium by two alternative routes. The first was traditional processing of clarified feed material by packed bed ion exchange chromatography; the second was direct adsorption of crude, unclarified feed material on an expanded bed of STREAMLINE SP. The complete scheme of the traditional route consisted of centrifugation (20 000 g for 1 hour at +4 °C), filtration, initial purification on a S Sepharose High Performance column (3.5 x 10 cm) and final purification on a Q Sepharose High Performance column (3.5 x 10 cm) or Mono Q HR 10/10. The complete scheme of the alternative route consisted of direct capture of the unclarified culture medium onto STREAMLINE SP followed by final purification on Mono Q.

In the expanded bed route, the crude feed material was applied to a STREAMLINE 50 column (50 mm i.d.) containing 300 ml of STREAMLINE SP adsorbent, corresponding to a sedimented bed height of 15 cm. The flow velocity during expansion/equilibration, adsorption and wash was 300 cm/h. The buffer used during expansion/equilibration and wash was 25 mM MES-NaOH, 1 mM EDTA, pH 6.25. Desorption of IL-1ra from the adsorbent was performed with downward flow in sedimented mode using a linear gradient of NaCl from 0 to 0.5 M in 25 mM MES-NaOH, 1 mM EDTA, pH 6.25. The flow velocity during elution was 100 cm/h. Fractions containing IL-1ra were pooled and subjected to final purification on a Mono Q HR 10/10 column. Table 34 summarizes the complete purification sequence by both the traditional route and the expanded bed route.

Table 34. Summary of results from purification of recombinant IL-1ra (DoB 0039) by two alternative routes.

Purification step	Traditional route		Expanded bed route	
	Purity (%)	Recovery (%)	Purity (%)	Recovery (%)
Crude starting material	14	100	14	100
Centrifugation	n.d.	n.d.	-	-
Filtration	14	96	-	-
Cation exchange	94	84	90-92	85
Anion exchange	98	74	98	73

A final purity of 98%, and an overall recovery of 74%, was achieved when clarified start material was purified by cation exchange chromatography on S Sepharose High Performance followed by anion exchange chromatography on Q Sepharose High Performance. When crude, unclarified material was applied directly on STREAMLINE SP, a purity of 90–92% with a recovery of 85% was achieved, very close to that obtained by centrifugation, filtration and S Sepharose High

Performance chromatography. After subjecting the pooled fractions from STREAMLINE SP to anion exchange chromatography, a final purity of 98% was reached, identical to that obtained with the traditional route. It was concluded that centrifugation, filtration and cation exchange chromatography on S Sepharose High Performance could all be replaced by a single adsorption step on STREAMLINE SP.

Expanded Bed Adsorption in Capture from Yeast Fermentation Cultures

This section contains applications of adsorption from yeast fermentation cultures, including examples where the target molecule is accumulated intracellularly or secreted into the cell culture broth.

Recovery of glucose-6-phosphate dehydrogenase from *S. cerevisiae* homogenate by expanded bed anion exchange adsorption

Glucose-6-phosphate dehydrogenase (G6PDH) was purified from crude, unclarified homogenate of bakers' yeast by the use of expanded bed anion exchange adsorption (34).

G6PDH, an intracellular enzyme found in baker's yeast, was released from the yeast cells by bead milling. The resulting 50% (w/v) wet weight homogenate was diluted (1:2) with 50 mM sodium phosphate, pH 6.0, to a total protein concentration of 12.8 mg/ml (2.69 units G6PDH/ml; 0.21 units G6PDH/mg protein). The biomass dry weight of the final 25% (w/v) homogenate was 6.5% (w/w). The viscosity was 5.0 mPa (4 °C, at a shear rate of 106 s-1) and the conductivity was 7.4 mS/cm (4 °C).

The 25% (w/v) unclarified cell homogenate was applied on STREAMLINE DEAE adsorbent, expanded and equilibrated with 50 mM sodium phosphate, pH 6.0, in a STREAMLINE 50 column (50 mm i.d.). The column contained 435 ml of sedimented adsorbent providing a sedimented bed height of 22 cm. The total activity of G6PDH loaded onto the bed corresponded to 43% of the equilibrium capacity of the column. The flow velocity during expansion/equilibration was 196 cm/h, causing the bed to expand to a height of 44.5 cm. The expanded bed height was maintained constant throughout feed application and wash by continuous adjustment of the flow velocity. The position of the float of a rotameter positioned in the inlet to the bed was used to estimate the location of the top of the expanded bed. Non-adsorbed components were washed out from the expanded bed using 25% (v/v) glycerol in 50 mM sodium phosphate, pH 6.0. This wash solution, having a viscosity similar to the feed-stock, allowed complete removal of residual particulate material from the bed by passage of a single expanded bed volume through the bed. The glycerol solution was subsequently removed from the bed by

washing with buffer and the bed was converted to a packed configuration prior to elution. The enzyme was desorbed from the bed by a three-step elution scheme using a flow velocity of 200 cm/h.

The first step was elution by 0.05 M NaCl (50 mM sodium phosphate, pH 6.0), the second was elution by 0.15 M NaCl (50 mM sodium phosphate, pH 6.0), and the third step was 1.0 M NaCl (50 mM sodium phosphate, pH 6.0).

Table 35 summarizes the experimental results. The enzyme was recovered with a yield of 98% in the second elution step with 0.15 M NaCl. The purification factor was 12. The process time for the complete purification cycle was 3.3 hours (equilibration: 40 min; feed application: 30 min; wash: 60 min, elution: 70 min).

Table 35. Purification of G6PDH from yeast cell homogenate using expanded bed anion exchange adsorption.

Purification step	Volume (ml)	Liquid velocity (cm/h)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Yield of G6PDH (%)
Homogenate	1068	-	2873	13 670	0.21	(1.0)	(100)
Flow-through	1068	196-66	4	7273	-	-	0.14
Wash	550	66-122	5	4102	-	-	0.17
Eluate (1)	1300	200	42	258	-	-	1.46
Eluate (2)	2100	200	2819	1125	2.51	12.0	98.1
Eluate (3)	900	200	6	917	-	-	0.2
Total recovery (%)	-	-	100.0	100.0	-	-	-

To verify the function of the adsorbent after repeated use, STREAMLINE DEAE was subjected to 10 cycles of feed application, each followed by a wash with 25% (v/v) glycerol solution and a cleaning-in-place procedure consisting of: 1.0 M NaCl; 1.0 M NaOH; 1.0 M NaCl; 70% ethanol; and finally, adsorption buffer. Five sedimented bed volumes of each solution were applied at a flow velocity of 50 cm/h. The breakthrough capacity for BSA, and expansion characteristics, were determined before cycle 1 and after cycles 1, 5 and 10. Table 36 summarizes the results.

Table 36. Summary of results from a study on the re-useability of STREAMLINE DEAE.

	Start	1 cycle	5 cycles	10 cycles
Breakthrough capacity for BSA (mg BSA/ml sedimented bed)	58.7	59.2	58.2	58.6
Liquid velocity (cm/h) to give 2x bed expansion in aqueous buffer at 20 °C	200	205	196	203

The results indicated that the adsorbent could be reused for more than 10 cycles without compromising its function. No effect on the tested parameters could be seen over 10 cycles. It was also concluded that the resolution of proteins eluting from the adsorbent that had been regenerated by the CIP protocol was the same as that of the untreated adsorbent. No evidence was found of any carry-over or build-up of contaminants as a result of reusing the adsorbent in multiple cycles of operation.

Recovery of glucose-6-phosphate dehydrogenase from *S. cerevisiae* homogenate by expanded bed dye-ligand affinity adsorption

Purification of glucose-6-phosphate dehydrogenase (G6PDH) from crude, unclarified homogenate of baker's yeast was performed by the use of expanded bed affinity adsorption to Procion Red H-E7B immobilized onto STREAMLINE adsorbent (33).

The start material was the same type of yeast homogenate as described in the previous application for recovery of G6PDH by expanded bed adsorption on STREAMLINE DEAE (34). G6PDH was released from yeast cells by bead milling cells suspended in 50 mM sodium phosphate, pH 6.0. The resulting homogenate was diluted (1:2) with 50 mM sodium phosphate, pH 6.0, to a total protein concentration of 12.3 mg/ml (3.3 units G6PDH/ml; 0.27 units G6PDH/mg protein). The biomass dry weight of the final homogenate was 6.8% (w/w) and the viscosity was 5.0 mPa.

The unclarified yeast homogenate was applied to a STREAMLINE 50 column (50 mm i.d.) containing 420 ml STREAMLINE adsorbent, coupled with Procion Red H-E7B, corresponding to a sedimented bed height of 21 cm. The total activity of G6PDH loaded onto the bed corresponded to 23% of the estimated dynamic capacity of the column. Prior to feed application, the adsorbent was expanded and equilibrated with 50 mM sodium phosphate, pH 6.0, at a flow velocity of 152 cm/h causing the bed to expand to a height of 42.5 cm. The expanded bed height was maintained constant throughout feed application and wash by continuous adjustment of the flow velocity to compensate for the increased viscosity of the homogenate. Debris and non-adsorbed components were washed out from the expanded bed using 25% (v/v) glycerol in 50 mM sodium phosphate, pH 6.0. A single expanded bed volume of the wash solution allowed complete removal of residual particulate material from the bed. After the glycerol wash, the bed was allowed to settle and 50 mM sodium phosphate, pH 6.0 was used to wash out the glycerol from the bed at a flow velocity of 152 cm/h. The enzyme was desorbed from the bed by a multiple step elution procedure using downward flow at a flow velocity of 152 cm/h. The following elution steps were applied: (1) 50 mM triethanolamine-HCl, pH 8.0; (2) 100 mM NaCl in 50 mM triethanolamine-HCl, pH 8.0; (3) 10 mM NADP in 50 mM triethanolamine-HCl, pH 8.0; and (4) 2 M NaCl in 50 mM triethanolamine-HCl, pH 8.0.

G6PDH was recovered from the unclarified homogenate with a yield of 99% and an average purification factor of 103. No particulate material was found in the eluted enzyme, as judged by turbidometric and microscopic analysis. The process time for the complete purification cycle was 3 hours (40 min. equilibration; 30 min. feed application; 60 min. wash, 50 min. elution). Table 37 summarizes the experimental results.

Table 37. Purification of G6PDH from yeast cell homogenate using expanded bed dye-ligand affinity adsorption.

Purification step	Volume (ml)	Liquid velocity (cm/h)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor (%)	Yield of G6PDH
Homogenate	830	-	2732	10309	0.265	(1.0)	(100)
Flow-through	830	152-53	6	4814	-	-	-
Wash	440	53-90	3	3987	-	-	-
Eluate (1)	625	152	7	639	-	-	-
Eluate (2)	500	152	0	227	-	-	-
Eluate (3)	450	152	2698	99	27.25	103	98.8
Eluate (4)	500	152	121	521	-		-
Total recovery (%)	-	-	104	99.8	-	-	-

To verify the function of the adsorbent after repeated use, the Procion Red H-E7B coupled STREAMLINE adsorbent was subjected to 10 cycles of feed application, each followed by a wash with 25% (v/v) glycerol solution and a cleaning-in-place procedure using a cocktail containing 0.5 M NaOH and 4 M urea in 60% (v/v) ethanol. Five sedimented bed volumes of the cocktail were applied at a flow velocity of 50 cm/h. The breakthrough capacity for lysozyme, and expansion characteristics, were determined before cycle 1 and after cycles 1, 5 and 10. Table 38 summarizes the results. The results indicated that the adsorbent could be reused for more than 10 cycles without compromising its function. No effect on the tested parameters could be seen.

Table 38. Summary of results from a study on the re-useability of Procion Red H-E7B coupled STREAMLINE adsorbent.

	Start	1 cycle	5 cycles	10 cycles
Breakthrough capacity for lysozyme (mg lysozyme/ml sedimented bed)	21.2	20.3	22.1	20.4
Liquid velocity (cm/h) to give 2x bed expansion in aqueous buffer at 4 °C	153	154	153	152

Recovery of alcohol dehydrogenase (ADH) from *S. cerevisiae* homogenate by expanded bed hydrophobic interaction adsorption

The intracellular enzyme alcohol dehydrogenase (ADH) was purified from crude, unclarified homogenate of baker's yeast by the use of expanded bed hydrophobic interaction adsorption on STREAMLINE Phenyl (low sub) (prototype adsorbent) (68).

A 45% (w/v) yeast suspension in 20 mM potassium phosphate, pH 7, was homogenized in a high pressure homogenizer for 5 passages at 1.2 x 108 N m-2. The homogenate was diluted to a final total protein concentration of 10 mg/ml and brought to an ammonium sulphate concentration of 0.78 M in potassium phosphate buffer pH 7.

Before the expanded bed experiments on STREAMLINE Phenyl (low sub), method scouting was performed in packed bed mode using clarified feed material to investigate both anion exchange chromatography (DEAE Sepharose Fast Flow) and hydrophobic interaction chromatography (Phenyl Sepharose Fast Flow (low sub)) for their relative merits in capturing ADH. The anion exchange medium exhibited low capacity and poor selectivity for ADH when applied to the column in 20 mM potassium phosphate, pH 7 and was considered unsuitable for direct capture of the enzyme. The hydrophobic interaction medium exhibited good capacity and high selectivity for ADH, providing 93% yield and a purification factor of 7.6 when clarified feed was applied to a 5 ml packed bed of Phenyl Sepharose Fast Flow (low sub).

The expanded bed adsorption was performed in a STREAMLINE 50 column (50 mm i.d.) containing 300 ml of STREAMLINE Phenyl (low sub), corresponding to a sedimented bed height of 15 cm. Expansion, equilibration and wash were performed with 0.78 M ammonium sulphate in 20 mM potassium phosphate, pH 7, using a flow velocity of 200 cm/h. The same flow velocity was used during application of the crude, unclarified yeast homogenate to the expanded bed, which resulted in a degree of expansion of around 3.2. Desorption of the enzyme from the adsorbent was performed with downward flow in sedimented mode by step elution from 0.78 M ammonium sulphate to 0 M ammonium sulphate in start buffer. Fig. 35 shows the expanded bed adsorption step on STREAMLINE Phenyl (low sub).

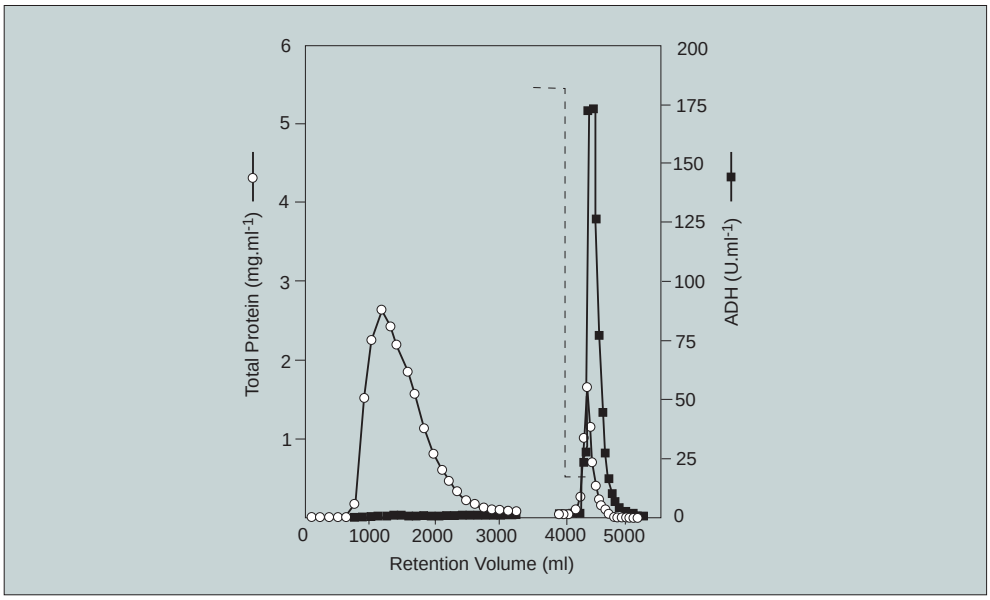


Fig. 35. Purification of ADH from yeast homogenate by expanded bed adsorption on STREAMLINE Phenyl (low sub). (----------) represents step elution from 0.78 M ammonium sulphate to 0 M ammonium sulphate. (Reproduced with permission from ref. 68).

The expanded bed adsorption on STREAMLINE Phenyl (low sub) was compared with packed bed adsorption using Phenyl Sepharose Fast Flow (low sub) packed in an XK 50/40 column (50 mm i.d.) to a final bed height of 15 cm. Table 39 presents the results from successive loadings onto both the expanded and the packed bed in terms of ADH eluted as percent yield of total loaded. In the expanded bed route, 95% of the total ADH loaded was recovered compared with 85% for the packed bed. The packed bed fouled more rapidly than the expanded bed for the same volume of material processed, indicating that the high speed centrifugation (38 000 g for 60 minutes) employed prior to packed bed adsorption was insufficient to completely clarify the feed stock.

Table 39. Variation in yield of ADH as a function of load volume and number of CIP cycles. One CIP cycle was performed after each adsorption cycle.

Cumulative volume loaded (bed volumes)	ADH Yield Expanded Bed (%)	ADH Yield Packed Bed (%)
1	95	85
3	114	71
5	105	58

Purification of recombinant Aprotinin Variants from *H. polymorpha* fermentation broth by expanded bed cation exchange adsorption

Expanded bed adsorption on STREAMLINE SP was used in the capture step during purification of two recombinant DesPro(2) aprotinin variants from *Hansenula polymorpha* fermentation broth (56).

Aprotinin is a bovine pancreatic trypsin inhibitor with a molecular weight of 6.5 kDa and an isoelectric point of 10.5. Aprotinin inhibits a range of proteases and has excellent potential as a therapeutic and diagnostic compound.

DNA sequences coding for the two aprotinin variants were expressed in the methylotropic yeast *Hansenula polymorpha*. The coding sequences were fused to the KEX2 recognition site of the *S. cerevisiae*-derived Mfα1 preproleader sequence, which causes secretion of the recombinant aprotinin variants into the culture broth.

The culture broth was prepared for expanded bed adsorption by dilution (1:1) with deionized water to a conductivity of 25 mS/cm, and by adjustment of pH to 3.5. The diluted culture broth had a biomass content of 5% dry weight.

A total of 6400 ml of crude, unclarified culture broth was applied to a STREAMLINE 50 column (50 mm i.d.) containing 300 ml of expanded STREAMLINE SP, corresponding to a sedimented bed height of 15 cm. Following feed application, unbound proteins and residual biomass were washed out from the bed in expanded mode. The flow velocity used during expansion/equilibration, adsorption and wash was 300 cm/h. The buffer used during expansion/equilibration and wash was 20 mM sodium citrate, pH 3.5. The expansion/equilibration with buffer at a flow velocity of 300 cm/h resulted in a three-fold expansion of the bed. Desorption of aprotinin from the adsorbent was performed with downward flow in sedimented mode applying a two-step elution procedure. In the first step, contaminating proteins were desorbed with 0.5 M NaCl in start buffer. The second step employed 0.9 M NaCl in start buffer to desorb the aprotinin. The matrix was finally regenerated with 2 M NaOH in sedimented mode.

The aprotinin-containing eluate from the STREAMLINE column was subjected to further purification by reversed phase chromatography. The eluate was supplemented with 0.1% (v/v) of TFA and applied to a RP 18 Lichroprep HPLC column (Merck, Darmstadt) at 2 cm/min without further pre-treatment. Pure aprotinin was eluted by a gradient from 0.1% TFA in aqueous solution to 50% of 0.1% TFA in 40% (v/v) isopropanol. The aprotinin-containing fractions were finally desalted by gel filtration on a Sephadex G-25 column and freeze-dried. Table 40 summarizes the results of the complete purification procedure.

Table 40. Summary of results from purification of aprotinin.

Purification step	Aprotinin concentration (mg/litre)	Purification factor	Yield (%)
Crude starting material	202	1	100
STREAMLINE SP	1412	3.8	76
RP 18 HPLC	719	5.45	36
Sephadex G-25	543	5.5	35

The purification step on the STREAMLINE SP adsorbent resulted in a 7-fold concentration and a 3.8-fold purification of aprotinin at a yield of 76%. Efficient removal of particulate material during the expanded bed adsorption step allowed direct application of the eluted material onto the RP-HPLC column without pre-treatment. The separation of incorrectly processed aprotinin was accomplished in the RP-HPLC step. HPLC, SDS-PAGE and N-terminal sequencing confirmed the fidelity and homogeneity of the isolated aprotinin.

Process for purification of recombinant human serum albumin from *P. pastoris* fermentation broth by expanded bed cation exchange adsorption

Expanded bed adsorption on STREAMLINE SP is used in the large scale production of recombinant human serum albumin (rHSA) (41).

In the process, rHSA is expressed in the yeast *Pichia pastoris* and secreted into the culture medium. At the end of fermentation, the culture medium (including host cells) is heat treated to inactivate proteases originating from the host. The heat treatment is followed by a two-fold dilution with distilled water to reduce conductivity, and adjustment of pH to 4.5.

The process was scaled up to about 1000 litres of culture medium giving a final volume of diluted unclarified culture medium of about 2000 litres. The diluted unclarified feed-stock was applied to a STREAMLINE CD column with an inner diameter of 1000 mm, containing 150 litres of STREAMLINE SP adsorbent corresponding to a sedimented bed height of 19 cm. The adsorbent was expanded and equilibrated with 50 mM acetate buffer, pH 4.5, containing 50 mM sodium chloride, prior to feed application. The feed was applied at a flow velocity of 100 to 250 cm/h under continuous stirring to prevent cell agglomeration. After feed application, the expanded bed was washed with 50 mM acetate buffer, pH 4.5, containing 50 mM sodium chloride, and subsequently the rHSA was desorbed from the adsorbent in sedimented mode using a downward flow velocity of 50 to 100 cm/h. The elution buffer was 100 mM phosphate buffer, pH 9, containing 300 mM sodium chloride.

The rHSA obtained from the expanded bed adsorption step is further purified by a combination of chromatography steps in packed bed configuration. Prior to this purification, the rHSA containing fraction from the STREAMLINE SP column is again heat treated in the presence of a reducing agent and stabilizers to reduce the degree of colouring of rHSA and to accelerate the conversion of dimer to monomer. Table 41 summarizes the outcome of four production scale runs up to the second heat treatment step, using 1 ton of crude unclarified culture medium in each run.

The average yield after the heat treatment at 68 °C for 30 minutes and the heat treatment with cysteine is 98.6% and 88.4% respectively. The total yield of the four runs is 87.1%, which is in good agreement with the results obtained at pilot scale on a STREAMLINE 50 column (50 mm i.d.).

It was concluded that the purity of rHSA obtained by this process (heat treatment - expanded bed adsorption) is almost comparable to that of rHSA obtained by a conventional 5-step process (filtration - ultrafiltration - heat treatment - ultrafiltration - cation exchange chromatography). Thus, the expanded bed process route reduces the number of steps from five to two, which shortens the processing time and increases the yield by 30%.

Table 41. Summary of results from four production scale runs from the purification of rHSA.

Run No.	Step	Volume (litres)	rHSA (g)	Yield (%)
1	culture medium	922	5868	100.0
	heat treatment (68 °C, 30 min)	1900	5399	92.0
	flow-through	6000	-	-
	eluate	200	-	-
	heat treatment with cysteine	62	4840	82.5
2	culture medium	943	6246	100.0
	heat treatment (68 °C, 30 min)	1960	6351	101.7
	flow-through	6400	-	-
	eluate	300	-	-
	heat treatment with cysteine	61	5674	90.9
3	culture medium	937	6200	100.0
	heat treatment (68 °C, 30 min)	1877	6261	101.1
	flow-through	5777	462	7.4
	eluate	200	-	-
	heat treatment with cysteine	111	5594	90.2
4	culture medium	916	6845	100.0
	heat treatment (68 °C, 30 min)	1885	6818	99.6
	flow-through	5885	-	-
	eluate	300	-	-
	heat treatment with cysteine	111	5804	84.8

Purification of a recombinant protein pharmaceutical from *P. pastoris* fermentation broth by expanded bed cation exchange adsorption

Expanded bed adsorption on STREAMLINE SP was evaluated by scientists at British Biotech plc, UK, as an alternative capture step in the manufacturing of a therapeutic protein for Phase II clinical studies (75a,b).

Material for phase I clinical studies was initially produced by a small pilot scale process starting with a fermentation volume of 60 litres. This process could not be scaled up for manufacturing the amounts required for the phase II clinical studies. Expanded bed adsorption on STREAMLINE SP was evaluated as an alternative to the clarification, concentration and initial cation exchange step in the established pilot scale process, see Fig. 36. Subsequent purification steps remained unchanged.

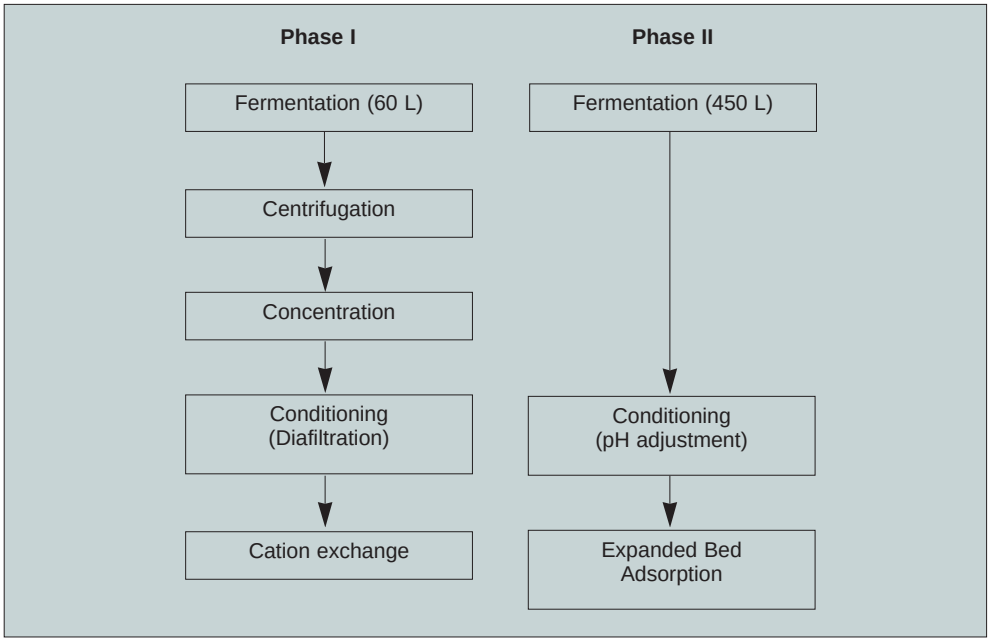


Fig. 36. Alternative purification routes for the recombinant protein pharmaceutical.

The product was secreted by the yeast *Pichia pastoris* with a biomass dry cell weight at harvest of 60 g/L. The molecular weight of the recombinant protein was 7.7 kDa and the isoelectric point was 4.5.

Method optimization was performed on a STREAMLINE 50 column (50 mm i.d.) containing 410 ml of STREAMLINE SP adsorbent, which corresponds to a sedimented bed height of 21 cm. Final production of phase II clinical material was performed on a STREAMLINE 200 column (200 mm i.d.) containing STREAMLINE SP adsorbent providing the same sedimented bed height of 21 cm.

The method development work included optimization of wash steps, replacement of gradient with stepwise elution and optimization of flow velocity and column loading to maximize product recovery. The effect of biomass dry weight on degree of expansion and bed stability was also examined. At biomass dry weights greater than 40 g/L, the bed was found to expand to the top of the column when the feed was applied at a flow velocity of 200 cm/h. At a biomass dry weight of 95 g/L, product recovery decreased to 66%. At biomass dry weights up to 65 g/L, recoveries were consistently greater than 82%. A biomass dry weight of 40 g/L was considered to offer the best balance in terms of feed-stock dilution, degree of bed expansion, overall processing time and product recovery.

The crude, unclarified feed was applied directly onto the expanded bed after dilution and adjustment of pH to 3.5. The flow velocity during feed application was 200 cm/h. The same flow velocity was applied during expansion/equilibration prior to feed application and during the wash in expanded mode after feed application. The buffer used during expansion/equilibration and wash in expanded mode was 50 mM acetate buffer pH 3.5 containing 250 mM NaCl. The degeree of expansion before feed application was typically 2.3-2.4 times the height of the sedimented bed. After the initial wash out of residual cells and particulates in expanded mode, the bed was sedimented and washed with upward flow in two separate steps. In the first step (wash 1) the bed was washed with purified water to facilitate buffer exchange. In the second step (wash 2) the bed was washed with 50 mM phosphate buffer pH 6 containing 50 mM NaCl to selectively remove bound contaminants prior to product elution.
Elution was performed at a flow velocity of 200 cm/h using upward flow in sedimented bed mode. Fig. 37 shows a typical chromatogram from a run on the STREAMLINE 50 column. The chromatograms from the phase II production runs looked identical.

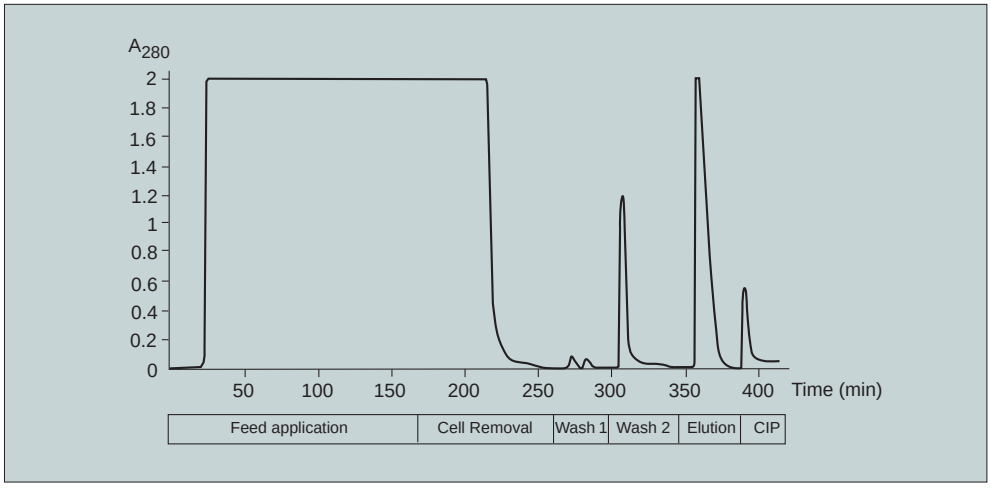


Fig. 37. Chromatogram from a typical process run on the STREAMLINE 50 column (Reproduced with permission, from ref. 75a)

After each purification cycle, the column was subjected to cleaning-in-place using a solution of 0.5 M NaOH and 1.0 M NaCl. The cleaning solution was initially pumped through the sedimented bed for 30 minutes using downward flow. The flow velocity was 100-150 cm/h. Then the flow direction was reversed for 30 minutes with the adaptor in its uppermost position. The bed was then left in contact with the cleaning solution for 1 hour (method development with the STREAMLINE 50 column) or overnight (phase II production with the STREAMLINE 200 column).

No effect on the dynamic binding capacity of the STREAMLINE SP could be detected for adsorbent that had undergone 12 process and CIP cycles.

Table 42 summarizes the results from a typical process run on the STREAMLINE 50 column using a feed-stock with a biomass dry weight of 30 g/L. The product was recovered with a yield of 84% and a 6-fold concentration. The majority of the protein contaminants were separated from the product as determined by RP-HPLC. Potentially detrimental proteases were also selectively removed from the product stream. This was verified by comparing mass spectra of the feed-stock, wash 2 and eluate, incubated for 1 week at +25 °C, with controls stored at -70 °C. The mass spectra showed high intrinsic stability for the product in the eluate while proteolytic degradation of the product in the feed-stock and wash 2 was clearly evident.

Table 42. Typical results from a process run on the STREAMLINE 50 column.

	Volumes (ml)	Total product (mg)	Yield (%)
Fermentation	6500		
Adsorbent	410		
Feed-stock	18500	3300	(100)
Flow-through	24500	2	0.06
Wash 1	1000	<1	<0.03
Wash 2	4200	55	1.7
Eluate	1050	2773	84

A number of analytical techniques were employed to demonstrate that the end product produced by the expanded bed route was of equivalent quality to the product produced by the established pilot scale process. All results indicated that the products from the two different purification routes were indistinguishable in terms of purity, identity and biological activity. The use of this material in phase II clinical trials resulted in British Biotech being the first company to file an IND with expanded bed adsorption central to the purification process.

Expanded Bed Adsorption in Capture from Mammalian Cell Cultures

This section contains some applications on expanded bed adsorption from crude, unclarified mammalian cell culture broth based on CHO cells, myeloma cells and hybridoma cells.

Recovery of Nerve Growth Factor from CHO cell culture broth by expanded bed cation exchange adsorption

Expanded bed adsorption on STREAMLINE SP was evaluated as an alternative capture step in the manufacturing of recombinant human Nerve Growth Factor (67). The work was performed by Genentech Inc., So. San Francisco, CA, USA, in collaboration with Pharmacia Biotech.

The established process consisted of cell harvest by tangential flow filtration, initial capture by packed bed cation exchange chromatography, followed by three other chromatography steps and final product formulation. Expanded bed adsorption on STREAMLINE SP was evaluated as an alternative to the cell harvesting and the initial cation exchange steps (see Fig. 38).

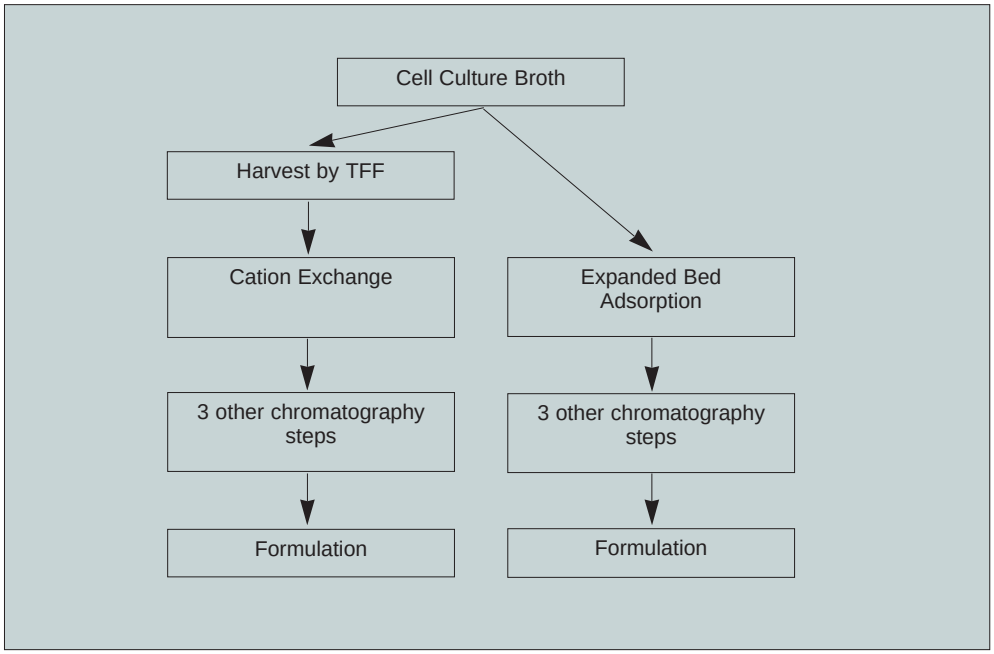


Fig. 38. Alternative purification paths for the recombinant human Nerve Growth Factor.

The recombinant human Nerve Growth Factor (rhNGF), which was produced in CHO cells, is a 26 kDa homodimer consisting primarily of a 118 amino acid residue monomer. The isoelectric point is >9.

Method scouting was performed on a 1.0 x 4.5 cm packed bed of STREAMLINE SP using clarified feed-stock. After having defined suitable conditions for binding and elution, method optimization in expanded bed mode was performed on a STREAMLINE 25 column (25 mm i.d.). The method was finally scaled up to a STREAMLINE 200 column (200 mm i.d.) containing 3 litres of STREAMLINE SP adsorbent corresponding to a sedimented bed height of approximately 10 cm.

The method optimization studies revealed that by applying the feed at a temperature of 37 °C, it was possible to increase flow velocity and still achieve high binding capacity. The optimum flow velocity was defined as 375 cm/h, which was the flow velocity applied during bed expansion/equilibration, feed application and wash. The binding capacity for rhNGF was 10 mg/ml adsorbent under those conditions. The crude, unclarified feed was applied directly onto the expanded bed after adjustment of pH to 5.7. The buffer used during expansion/equilibration and wash was 25 mM MES/NaMES, 0.3 M sodium acetate, pH 6.0. Elution was performed at a flow velocity of 100 cm/h using downward flow in sedimented bed mode. The elution buffer was 25 mM MES/NaMES, 1 M sodium acetate, pH 6.0. After each purification cycle, the column was subjected to cleaning-in-place using a solution consisting of 1.0 M NaOH and 0.5 M NaCl.

Figure 39 shows a chromatogram from a purification cycle on the STREAMLINE 25 column and Table 43 summarizes the results after scale up to the STREAMLINE 200 column. Recovery of rhNGF ranged from 93% to 100%. The purification factor was at least 11-fold. The concentration factor was 30-50 fold.

The product pool from the STREAMLINE 200 column was further processed through the established process minus the cation exchange chromatography step. The final product was compared with the product produced by the established process. The product processed by expanded bed adsorption was equivalent to that processed using the existing process with respect to product quality. Purity was determined by SDS-PAGE, RP-HPLC, and other assays that detect contaminant levels. It was concluded that expanded bed adsorption met all the criteria for implementation in manufacturing.

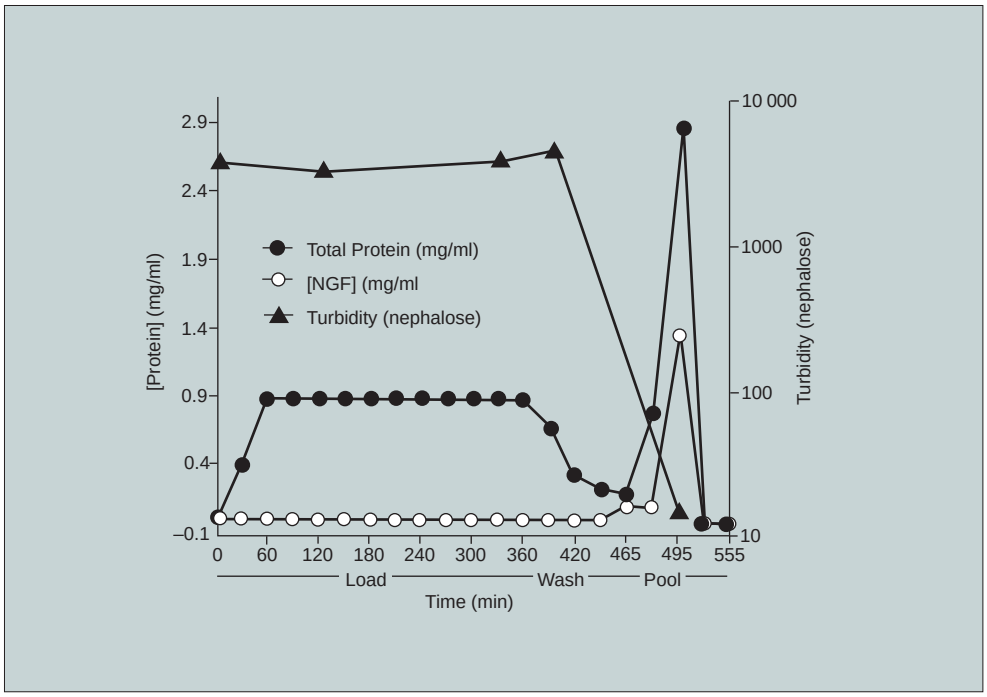


Fig. 39. Expanded bed adsorption of rhNGF on STREAMLINE SP in a STREAMLINE 25 column. (Work by Genentech Inc., So. San Francisco, CA, USA, in collaboration with Pharmacia Biotech.)

Table 43. Summary of results from the recovery of rhNGF on STREAMLINE SP in a STREAMLINE 200 column.

Adsorbent volume	3 L
Feed-stock volume	400 L
Cell viability	52%
Packed cell volume	2.7%
Degree of expansion (maximum)	5.4
Concentration factor	30
Purification factor	11
Yield	100%
Cycle time	6 hours

Recovery of a recombinant monoclonal antibody from CHO cell culture broth by expanded bed cation exchange adsorption at large scale

Expanded bed adsorption on STREAMLINE SP was used to capture a recombinant monoclonal antibody from crude, unclarified CHO cell culture suspension at 12000 litres scale (66). The work was performed by Genentech Inc., So. San Francisco, CA, USA, in collaboration with Pharmacia Biotech.

The recombinant monoclonal antibody was produced in CHO cells at an expression level of 500-600 mg/L. It had an isoelectric point of 9.1 and therefore bound efficiently to a cation exchange resin at acidic pH values. Optimal binding and elution conditions were defined using clarified harvested cell culture fluid in packed bed mode in a 1 x 10 cm column. The conductivity in the cell culture fluid was approximately 15 mS/cm. To achieve good binding, the cell culture fluid had to be diluted to around 6 mS/cm before application to the STREAMLINE SP adsorbent. Optimal binding was achieved at around pH 5.5.

After the method scouting in packed bed mode, the method was optimized in expanded mode using a STREAMLINE 25 column (25 mm i.d.). The column contained 75 ml of STREAMLINE SP corresponding to a sedimented bed height of 15 cm. The crude, unclarified cell culture suspension was applied directly onto the STREAMLINE SP adsorbent using on-line dilution to reduce conductivity immediately before the feed entered the column. In the initial experiments, plain water or starting buffer was used as diluent. Cell viability in the cell culture was 66%, which had been reduced to 20% at the time when the feed had passed through the column. Cell viability was measured as the activity of lactate dehydrogenase (LDH) in the cell culture suspension. The decrease in cell viability indicated that cells lysed as a consequence of changed osmotic pressure during dilution. Extensive expansion of the bed during feed application gave further evidence for cell lysis causing increased viscosity due to release of nucleic acids.

Further experiments using 200-300 mM glucose as diluent confirmed that extensive cell lysis occured if the cells were not protected against change in osmotic pressure during dilution. Using 200-300 mM glucose as diluent gave higher cell viability, lower degree of expansion, higher product yield and also made it easier to restore bed expansion characteristics by cleaning-in-place.

After method optimization in the STREAMLINE 25 column, the method was scaled-up 2200-fold, allowing the purification of recombinant monoclonal antibody from 12000 litres of cell culture suspension.

Using on-line dilution with 200 mM glucose, unclarified cell culture suspension was applied directly onto a STREAMLINE 1200 column (customized column with 1200 mm i.d.) after adjustment of pH to 5.4. The column contained 170 litres of STREAMLINE SP corresponding to a sedimented bed height of 15 cm.

The buffer used during expansion/equilibration and wash was 20 mM MES, pH 5.4. The flow velocity during expansion/equilibration, feed application and wash was 300 cm/h. Elution was performed at a flow velocity of 100 cm/h using downward flow in sedimented bed mode. The elution buffer was 0.25 M tetramethyl ammonium chloride (TMAC), 1 M NaCl.
After the purification cycle had been completed, the column was immediately subjected to cleaning-in-place using a solution of 0.5 M NaOH and 1 M NaCl. The solution was pumped through the bed with upward flow at a flow velocity of 100 cm/h. The adaptor was positioned at three times the sedimented bed height. After approximately two hours, when the main contaminant peak had been eluted from the column, the CIP solution was recirculated through the bed for another 6 hours.

Table 44 summarizes the results from processing of 7324 litres of unclarified cell culture suspension on the STREAMLINE 1200 column. The process resulted in complete removal of cellular mass and a five-fold concentration of the antibody. Furthermore, 99% of the antibody was recovered.

Table 44. Summary of results from the recovery of a recombinant monoclonal antibody on STREAMLINE SP in a STREAMLINE 1200 column.

Volume (L)	
Adsorbent	170
Feed-stock (undiluted)	7324
Feed-stock (diluted)	17396
Wash	4807
Elution	1409
CIP	4800
Time (hours)	
Feed application	5.5
Wash	2.4
Elution	1.3
CIP	6
Cell density in feed-stock (cells/ml)	13.8×10^5
Cell viability in feed-stock (%)	52
Challenge (mg antibody/mg adsorbent)	21.5
Concentration factor	5
Yield (%)	99

Recovery of an antibody from CHO cell culture broth by expanded bed cation exchange adsorption

A process has been described for expanded bed adsorption on STREAMLINE SP to capture an antibody from crude, unclarified CHO cell culture broth (25).

Two pilot runs were performed by applying unclarified serum-free whole culture broth to a 50 mm inner diameter column containing 170 ml of STREAMLINE SP adsorbent corresponding to a sedimented bed height of 8.6 cm. Prior to loading of

feed-stock, 25 mM MES buffer, pH 5.4 was run through the column to equilibrate the adsorbent and gradually expand the bed to a final height of 2.4 times its sedimented height.

The culture broth was diluted three-fold on-line by a separate buffer feed to reduce the ionic strength and pH to a level suitable for adsorption to the STREAMLINE SP adsorbent. On-line dilution was applied to minimize exposure of cells to reduced pH and osmolality which could cause cell lysis. The combined flow velocity of the two feeds was such that approximately the same 2.4 times expansion of the bed was maintained throughout adsorption. This corresponded to an average flow velocity of 135 cm/h through the bed in the first run. In the second run, the required average flow velocity to generate the same degree of expansion was 144 cm/h. The cell culture broths were stored for 2 and 3 days respectively before processing, which reduced the cell load by partial sedimentation. The cell concentration in the undiluted culture broth was 5 x 10^4 cells per ml at the start of the process.

Following adsorption, the expanded bed was washed with approximately 24 sedimented bed volumes of equilibration buffer to remove unadsorbed protein and any remaining cells from the bed.

Following the wash, the bed was allowed to sediment and the antibody was eluted with a linear gradient from 40 to 400 mM NaCl in 25 mM MES buffer, pH 5.4. In the second run, the pH of the elution buffer was increased to 6.4 to tighten the product peak and reduce the number of fractions required to recover the antibody.

After elution, the column was stripped with 1 M NaCl/25 mM MES buffer to remove any protein still adsorbed.

Finally, the adsorbent was cleaned with 1 M NaOH, followed by 1 M NaCl (3 bed volumes each), then distilled water to bring the pH to neutral.

Table 45 summarizes the results from the two pilot runs.

The antibody elution profile in the first run exhibited a broad peak with a long tail. The pooled fractions contained 69.4% of the antibody in the culture broth. Another 5.1% was found in the column strip effluent, which means that the overall antibody recovery was 74.5%.

In the second run, the antibody peak was much sharper due to the increased pH of the elution buffer. Only 0.4% of the antibody was found in the column strip effluent. Due to the higher antibody load in the second run, breakthrough of antibody was observed in the flow-through but nearly 100% of the adsorbed antibody was recovered.

A 19-fold reduction of volume and a 6-fold increase in antibody purity was achieved in the first run. In the second run, the product volume was 47 times less than the whole broth and the purification factor was 7.3.

Table 45. Summary of results from two pilot runs of expanded bed adsorption on STREAMLINE SP.

Run No.	Step	Volume (litres)	Antibody conc.(mg/L)	Total protein conc. (mg/L)	mg antibody per mg total protein
1	whole broth	26	4.98	260	0.0192
	diluted feed	78	1.44	100	0.0144
	flow-through	78	0.053	87.5	6.06×10^{-4}
	pooled eluate fractions	1.395	64.4	556.8	0.116
	column strip effluent	0.7	9.4	530	0.0177
2	whole broth	36	7.43	270	0.0275
	diluted feed	108	2.14	90	0.0238
	flow-through	108	0.38	70.2	5.84×10^{-3}
	pooled eluate fractions	0.765	294.9	1473.5	0.200
	column strip effluent	0.7	1.18	410	2.88×10^{-3}

The operating cost for the expanded bed adsorption process was compared with an alternative process consisting of a microfiltration step and an ultrafiltration step. Labour costs, filter/adsorbent costs and reagents cost were calculated for processing of 250 L of whole cell culture broth. The labour cost was found to be four times lower for the expanded bed process. Assuming one set of filters would be sufficient for 100 filtration batches and the given adsorbent volume would be sufficient for only 10 batches, the calculated filter cost was still twice as high as the adsorbent cost. The overall operating cost for the expanded bed process was estimated to be 3.6 times less than the filtration process. Furthermore, it was concluded that the expanded bed process offers further advantages due to the partial purification achieved and the higher recovery compared with the alternative process.

Purification of a humanized IgG_4 antibody from myeloma cell culture broth by expanded bed affinity adsorption

A humanized IgG_4 monoclonal antibody was purified from a myeloma cell culture by expanded bed adsorption on STREAMLINE rProtein A (65). The work was performed by Pharmacia Biotech, Uppsala, Sweden, in collaboration with Celltech Biologics Plc., UK.

Crude, unclarified cell culture broth from the fermentor was applied directly onto a STREAMLINE column containing STREAMLINE rProtein A at an amount corresponding to a sedimented bed height of 15 cm. The temperature of the cell culture broth was 37 °C at the time of feed application. Prior to feed application, the bed was expanded and equilibrated with 50 mM glycine-glycinate, pH 8,

containing 250 mM NaCl. The flow velocity during expansion/equilibration and feed application was 300 cm/h. After the sample had been applied, the expanded bed was washed with starting buffer until the signal from the UV monitor returned to baseline. This was followed by an additional 10 sedimented bed volumes of starting buffer. Elution was performed with downward flow in sedimented mode at a flow velocity of 100 cm/h. The elution buffer was 0.1 M glycine, pH 3.0.

After elution, the bed was subjected to cleaning-in-place by passing 2 sedimented bed volumes of 6 M guanidine hydrochloride through the bed using a flow velocity of 100 cm/h in expanded bed mode.

Two subsequent runs, as described above, were performed at laboratory scale by applying 1.5 litres of cell culture broth to a STREAMLINE 25 column (25 mm i.d.) containing 75 ml of STREAMLINE rProtein A. The process was then scaled up to pilot scale by applying 93 litres of cell culture broth to a STREAMLINE 200 (200 mm i.d.) containing 4.7 litres of STREAMLINE rProtein A.

Fig. 40 shows chromatograms from laboratory scale and pilot scale runs on STREAMLINE 25 and STREAMLINE 200 respectively.

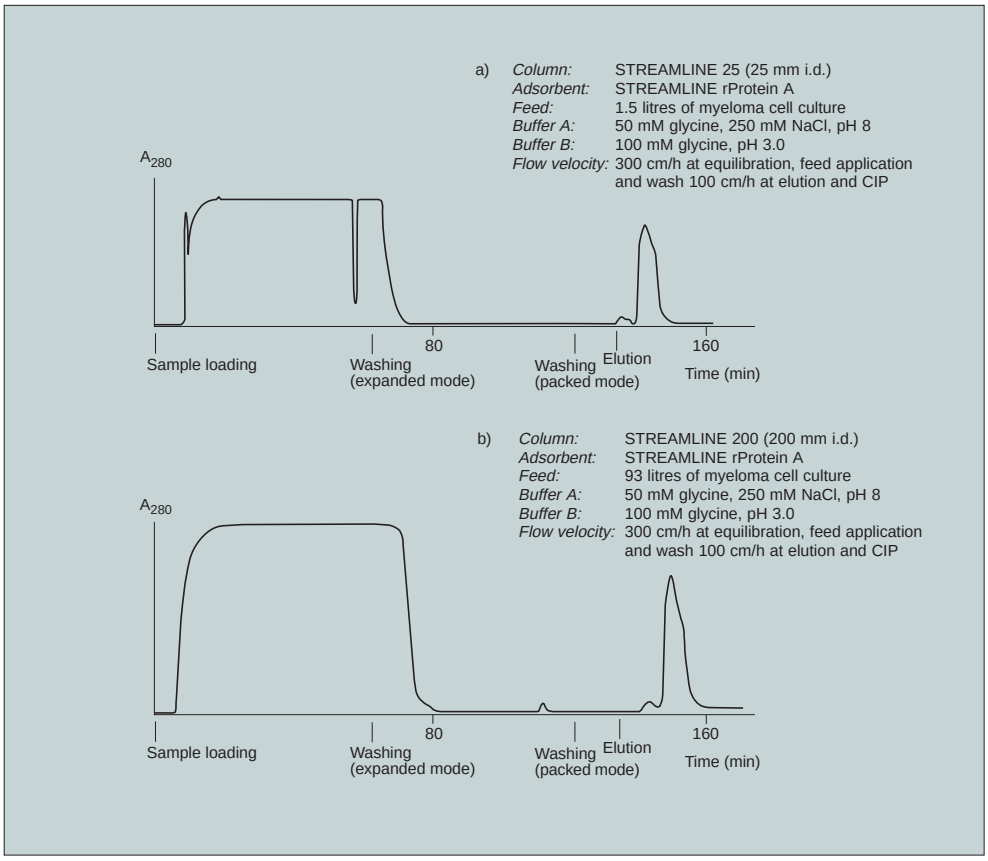

a)
Column: STREAMLINE 25 (25 mm i.d.)
Adsorbent: STREAMLINE rProtein A
Feed: 1.5 litres of myeloma cell culture
Buffer A: 50 mM glycine, 250 mM NaCl, pH 8
Buffer B: 100 mM glycine, pH 3.0
Flow velocity: 300 cm/h at equilibration, feed application and wash 100 cm/h at elution and CIP
A280
80
160
Sample loading
Washing (expanded mode)
Washing (packed mode)
Elution
Time (min)
b)
Column: STREAMLINE 200 (200 mm i.d.)
Adsorbent: STREAMLINE rProtein A
Feed: 93 litres of myeloma cell culture
Buffer A: 50 mM glycine, 250 mM NaCl, pH 8
Buffer B: 100 mM glycine, pH 3.0
Flow velocity: 300 cm/h at equilibration, feed application and wash 100 cm/h at elution and CIP
A280
80
160
Sample loading
Washing (expanded mode)
Washing (packed mode)
Elution
Time (min)

Fig. 40. Laboratory (a) and pilot scale (b) purification of a monoclonal IgG_4 antibody on STREAMLINE rProtein A. (Work by Pharmacia Biotech in collaboration with Celltech Biologics Plc.)

Table 46 summarizes the results from two runs at laboratory scale on STREAMLINE 25 and the pilot scale run on STREAMLINE 200. The purity as determined by SDS PAGE was high and consistent between the different runs. Very low levels of BSA, transferrin and DNA were found in the eluate. The antibody was recovered at high yield with a 15-fold concentration.

Table 46. Summary of results from the laboratory scale and pilot scale purification of humanized monoclonal IgG_4 by expanded bed adsorption on STREAMLINE rProtein A.

	STREAMLINE 25		STREAMLINE 200	
	run 1	run 2		
Adsorbent (L)	0.075	0.075	4.7	
Feed				
Volume (L)	1.5	1.5	93	
Conc. IgG_4 (mg/ml)	0.407	0.407	0.407	
Amount IgG_4 (mg)	611	611	37900	
Eluate				
Volume (L)	0.0912		0.0912	6.134
Conc. IgG_4 (mg/ml)	7.41	7.46	7.68	
Amount IgG_4 (mg)	676	677	47109	
BSA (ng/mg IgG_4)	8.6	25	10	
Transferrin (ng/mg IgG_4)	< 0.2	< 0.4	< 0.4	
DNA (pg/mg IgG_4)	N/A	N/A	< 0.26	
Yield (%)	111	111	124	

Purification of a murine IgG_1 antibody from hybridoma cell culture broth by expanded bed affinity adsorption

A murine IgG_1 monoclonal antibody was purified from a hybridoma cell culture by expanded bed adsorption on STREAMLINE rProtein A (73).

A mouse hybridoma cell line producing an IgG_1 monoclonal antibody was cultivated in a spinner system, a 5 L bioreactor and a 100 L bioreactor. The cells were cultivated on a serum-free medium. The cell density was 3 x 10^6 living cells/ml. The product titre was in the range 70 to 100 mg IgG_1/L.

Method scouting was performed on a small packed bed of STREAMLINE rProtein A using clarified feed material.

After having defined optimal loading conditions (pH, ionic strength and flow velocity) on the small packed bed, laboratory scale expanded bed experiments were performed on a STREAMLINE 25 column (25 mm i.d.). The STREAMLINE 25 column contained 75 ml of STREAMLINE rProtein A adsorbent corresponding to a sedimented bed height of 15 cm. The column was operated in a semi-automated system based on the programmable fraction collector GradiFrac and the piston pump P-50 (Pharmacia Biotech).

The method was finally scaled up to pilot scale using a STREAMLINE 200 column (200 mm i.d.) containing 5 litres of STREAMLINE rProtein A corresponding to a sedimented bed height of 15 cm. The STREAMLINE 200 column was operated by a peristaltic pump.

Bed expansion/equilibration, feed application and wash were performed at an upward flow velocity of 300 cm/h. The crude, unclarified feed was applied directly onto the expanded bed after adjustment of pH. The buffer used during expansion/equilibration and wash was 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl. Elution was performed at a flow velocity of 100 cm/h using downward flow in sedimented bed mode. The elution buffer was 50 mM phosphate, 50 mM sodium citrate, pH 4.5, containing 150 mM NaCl. A concentrated basic buffer (0.5 M Tris-HCl, 150 mM NaCl, pH 8.6) was introduced into the eluate to adjust pH to a more moderate level.

Table 47 summarizes results from both laboratory and pilot scales. The results for the STREAMLINE 25 scale are average values from five purification cycles. The procedure was not run at maximum loading capacity. The capacity was 14 mg IgG_1/ml adsorbent as defined by frontal analysis of breakthrough during method scouting on the small packed bed column.

The purity of the eluted antibody was higher than 90% as determined by SDS PAGE. No contaminating albumin (initial concentration 500 mg/ml) remained in the eluate.

Table 47. Summary of results from laboratory scale and pilot scale purification of a monoclonal IgG_1 by expanded bed adsorption on STREAMLINE rProtein A.

	STREAMLINE 25	STREAMLINE 200
Adsorbent volume (L)	0.075	5
Feed volume (L)	~1	94.3
Conc. IgG_1 in feed (mg/ml)	~0.09	0.031
Amount IgG_1 applied (mg)	~90	2.93
Conc. IgG_1 in eluate (mg/ml)	~1.7	0.66
Concentration factor	~15	21
Yield (%)	95-100	90.6
Process time (minutes)	~100	115

During the laboratory scale runs on the STREAMLINE 25 column, the extent of cell damage caused by the P-50 piston pump, and by passage through the expanded bed, was investigated. Concentration of DNA, activity of lactate dehydrogenase (LDH), and the particle load in the flow-through were used as indicators on cell damage. Table 48 summarizes the results from those studies.

Despite the sensitivity of hybridoma cells to shear force, neither the high flow velocity with the piston pump nor the passage of the cells through the expanded bed caused any detectable cell damage. No increase in DNA concentration or LDH activity was found in the flow-through and wash fraction compared with the sample applied to the expanded bed. The increase in LDH activity in the cell culture broth during pH adjustment indicate that hybridoma cells are sensitive to such operations. Consequently, pH adjustment should be performed with great care with gentle stirring to prevent the formation of local extremes of pH. Particle measurement in the flow-through showed no increase of particles in the range 6–10 µm (dead cells) and only a slight decrease of total cell amount in the range 10–60 µm (living cells). No particles were found in the eluate. Furthermore, the DNA concentration and LDH activity were greatly reduced in the eluate from the expanded bed compared with the feed material.

Table 48. Detection of cell damage by measuring DNA concentration, LDH activity and particle load.

	DNA (ng/ml)[1]	LDH activity (nkat/L)[2]	Particle load (counts/ml)	
			ø 6-10 µm	ø 10-50 µm
Cell culture broth, pH 7.2	1917	1939	3.80×10^5	2.67×10^6
Feed, pH 8.0 (approx. 1 litre)	1926	3120	3.85×10^5	2.55×10^6
Passage total (flow-through and wash)	2003	3000	4.00×10^5	2.52×10^6
Eluate	0	45	0	0

[1] Detection limit: 1 ng/ml

[2] Maximum LDH limit after destruction of all cells: approx. 15000 nkat/L (nkat = nanokatal)

Purification of a murine IgG_{2a} antibody from hybridoma cell culture broth by expanded bed affinity adsorption

A murine IgG_{2a} monoclonal antibody was purified from a hybridoma cell culture by expanded bed adsorption on STREAMLINE rProtein A (64).

The monoclonal antibody was produced in a continuous culture using immobilized hybridoma cells. The average cell concentration in the culture broth was 1 x 106 cells/ml. The product titre was in the range 14 to 50 mg IgG_{2a}/L.

Method scouting was performed on a 1 ml packed bed of STREAMLINE rProtein A using clarified feed material. Optimum pH conditions for binding and elution were defined as pH 7.0 and pH 4.0 respectively.

Laboratory scale expanded bed experiments were performed on an XK 16/40 column (Pharmacia Biotech), a column designed for packed bed chromatography. To improve flow distribution in expanded mode, a 1 cm bed of non-porous glass ballotini (3 mm diameter) was packed at the bottom inlet. The column contained

20 ml of STREAMLINE rProtein A adsorbent corresponding to a sedimented bed height of 10 cm. Initial experiments with cell-free hybridoma supernatant revealed that the capacity for the IgG_{2a} antibody was 14 mg/ml adsorbent.

The method was scaled up in two steps. The first scale up step was run on a STREAMLINE 25 column (25 mm i.d.) containing 50 ml of adsorbent corresponding to a sedimented bed height of 10 cm. The second was run on a STREAMLINE 50 column (50 mm i.d.) containing 150 ml of adsorbent, which corresponds to a sedimented bed height of 7.6 cm.

Bed expansion/equilibration, feed application and wash were performed at an upward flow velocity of 360 cm/h. The crude, cell-containing hybridoma broth was applied directly onto the expanded bed after adjustment of pH. The buffer used during expansion/equilibration and wash was 50 mM potassium phosphate, pH 7.0. Elution was performed both in expanded and packed bed mode using a flow velocity of 360 and 90 cm/h respectively. The elution buffer was 100 mM sodium citrate, pH 4.0. The eluate was collected in a vessel containing 1 M Tris/HCl, pH 8.0 for direct neutralization. After elution, the expanded bed was cleaned with five sedimented bed volumes of 2 M urea followed by five sedimented bed volumes of 1 M acetic acid.

Table 49 summarizes results from all three scales.

In one run on the STREAMLINE 25 column and in two runs on the STREAMLINE 50 column, elution was performed in an expanded mode of operation, which explains the lower concentration of antibody in the eluate.

The eluate from the different columns contained IgG_{2a} at very high purity as determined by SDS PAGE.

Fractions of the feed applied, the washing step and the final eluate were analysed for particle content with a Coulter Counter. A more than 100 fold clarification was achieved in addition to the very high purity of the antibody and the significant volume reduction.

Comparing the curent capacity with the capacity during a previous purification of the IgG_{2a} antibody by ion exchange expanded bed adsorption on STREAMLINE SP (35) shows a nearly 100-fold increase in capacity with STREAMLINE rProtein A. The reduced capacity on the STREAMLINE SP was due to the high conductivity (13 mS/cm) in the undiluted feed applied to the adsorbent. No such reduction in capacity is seen with the salt tolerant affinity adsorption on STREAMLINE rProtein A.

Table 49. Summary of results from three different scales of expanded bed adsorption of a monoclonal IgG_{2a} on STREAMLINE rProtein A.

	XK 16/40		STREAMLINE 25			STREAMLINE 50	
	Run 1	Run 2	Run 1	Run 2	Run 3[1]	Run 1	Run 2
Adsorbent volume (ml)	20	20	50	50	50	150	150
Feed volume (L)	10	10	20	20	20	60	60
Conc. IgG_{2a} in feed (mg/L)	18.9	13.8	34	50	50	40	29
Challenge (mg IgG_{2a}/ ml adsorbent)	8.8	6.9	13.6	20	20	16	11.6
Conc. IgG_{2a} in eluate (mg/L)	2500	1200	1199[2]	2084	2509	1197[2]	962[2]
Concentration factor	132	87	35	42	50	30	33
Yield (%)	126	87	95	78	84	83	82

[1] Flow velocity during expansion/equilibration, feed application and wash was 420 cm/h.

[2] Elution was performed in expanded mode using a flow velocity of 360 cm/h.

Expanded Bed Adsorption in Capture from Milk

This section contains two applications on expanded bed adsorption from milk; one from skimmed equine milk and the other one from milk of transgenic livestock.

Purification of lysozyme from equine milk by expanded bed cation exchange adsorption

Highly active pure lysozyme was purified from skimmed equine milk by expanded bed cation exchange adsorption on STREAMLINE SP (55).

The purpose was to develop a method to prepare lysozyme from milk that was faster than existing methods. In previously reported preparative techniques, caseins are first removed by salt or acid precipitation. This is followed by dialyzation and lyophilization steps and, finally, at least two chromatographic steps. These manipulations are time consuming and can result in significant inactivation of the enzyme. Therefore, direct capture of lysozyme from skimmed milk by expanded bed adsorption was evaluated as an alternative process.

Equine milk was defatted at +4 °C by centrifugation at 7500 g for 10 minutes. About two volumes of 60 mM Tris/HCl buffer were added to 1.6 litres of the milk to give a final Tris/HCl concentration of 20 mM. The pH was adjusted to 8.0 with HCl.

The defatted and diluted milk was applied to a STREAMLINE 50 column (50 mm i.d.) containing 300 ml STREAMLINE SP corresponding to a sedimented bed height of 15 cm. The flow velocity during expansion/equilibration, adsorption and wash was 300 cm/h. The buffer used during expansion/equilibration and wash was

20 mM Tris/HCl, 0.02% NaN3, pH 8.0. Desorption was performed with downward flow in sedimented mode using 20 mM Tris/HCl, 0.02% NaN3, pH 8.0, containing 1 M NaCl.

Figure 41 shows a chromatogram from the purification of skimmed milk on STREAMLINE SP in a STREAMLINE 50 column. The chromatogram showed two fractions, the flow-through fraction and the fraction eluted by the desorption buffer. The flowthrough fraction was slightly translucent, probably as a result of light scattering caused by casein micelles. Isoelectric focusing of samples from this fraction revealed a series of proteins with isoelectric points below pH 8. The elution fraction only contained proteins with isoelectric points above pH 8.0, including lysozyme having an isoelectric point above 10. A mixture of proteins with different molecular masses was detected in this fraction by SDS-PAGE. The elution fraction contained only 10% of the original amount of protein, but up to 89% of the lytic activity.

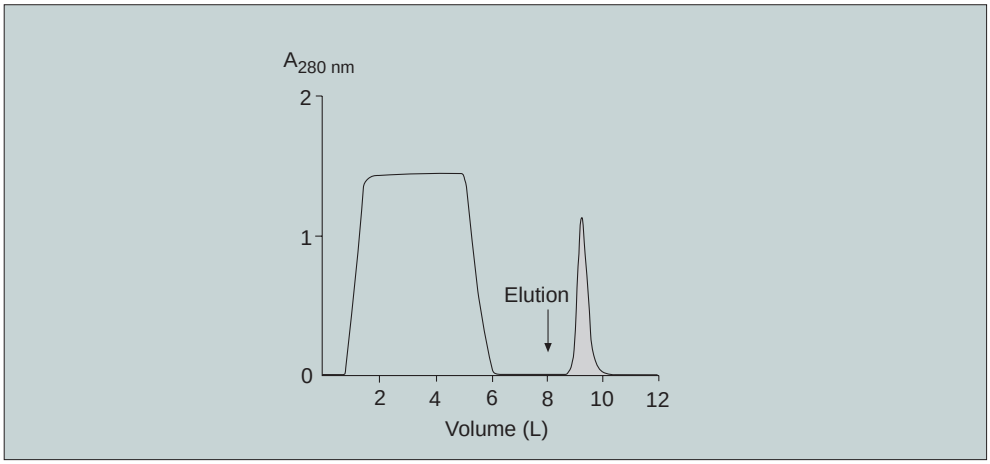


Fig. 41. Chromatogram showing the purification of lysozyme from skimmed equine milk on STREAMLINE SP in a STREAMLINE 50 column. The speckled area represents the lysozyme-containing fraction. (Reproduced with permission, from reference 55).

Following the Capture step on STREAMLINE SP, the material was further purified by hydrophobic interaction chromatography on Phenyl Sepharose 6 Fast Flow. The hydrophobic/hydrophilic nature of equine milk lysozyme is modified depending on the presence or absence of Ca^{2+} ions. The lysozyme was adsorbed to the Phenyl Sepharose 6 Fast Flow column in the presence of an excess of EDTA (50 mM Tris/HCl, 1 mM EDTA, pH 7.5) and desorbed with the same buffer when EDTA was replaced with Ca^{2+} ions. Table 50 summarizes the complete purification process. The peak eluted from the Phenyl Sepharose 6 Fast Flow column contained a single protein according to isoelectric focusing and SDS-PAGE. The molecular mass was determined as 14 400, which is consistent with that reported in the literature. The overall recovery of enzyme activity was greater than 80% of the activity originally found in the starting batch of skimmed milk.

Table 50. Summary of results from the purification of equine lysozyme from skimmed milk.

	Volume (ml)	Total protein (mg)	Total activity U (10^6)	Specific activity (U/mg)	Purification factor	Yield (%)
Skimmed milk	4990	42964	57.35	1335	1.0	100
STREAMLINE SP	560	4603	50.98	11075	8.3	88.9
Phenyl Sepharose 6 Fast Flow	2750	1547	46.88	26939	2.4	91.3

Purification of a recombinant protein from the milk of transgenic livestock by expanded bed anion exchange adsorption

The active sub-population of recombinant human protein C (rhPC) was purified from milk of transgenic pigs by expanded bed anion exchange adsorption on STREAMLINE DEAE (60).

Milk is a relatively complex mixture containing serum passover proteins such as albumin, broadly specific proteases, and caseins. The total protein content is 40 to 60 g protein/L. This complexity can make downstream processing difficult on a large scale. Recombinant proteins have been harvested from g/L levels in the milk of transgenic livestock using precipitation techniques in combination with chromatography. For example, multiple PEG precipitations in combination with ion exchange adsorption have been used to purify recombinant alpha-1 antitrypsin from the milk of transgenic sheep (76) and human protein C from the milk of transgenic swine (77). It has often been necessary to apply affinity chromatography to separate desired protein species from inactive recombinant protein sub-populations. Addition of PEG, the use of filtration or centrifugation to remove cells and protein precipitates, and subsequent immunoaffinity chromatography, all add significantly to the costs of producing therapeutic proteins from transgenic milk.

Downstream processing of rhPC from milk of transgenic pigs was simplified by an initial selective precipitation of a and b-caseins with low concentrations of Zn^{2+} ions. The whey, containing precipitated proteins, was applied directly to an expanded bed of STREAMLINE DEAE. The precipitated proteins passed through unhindered and eluted in the flow-through fraction while the rhPC was adsorbed onto the expanded bed. The addition of Zn^{2+} ions also increased the selectivity of the adsorption process due to reaction of Zn^{2+} ions with immature, inactive populations of rhPC. This specific interaction causes conformational changes within these populations, which allow them to pass through the bed unadsorbed while the active population of rhPC binds to the adsorbent.

Immediately after collecting the milk from transgenic lactating sows, it was diluted with 200 mM EDTA, pH 7.0, in a 1:1 ratio. This solubilizes the caseins, which exist as micelles. The milk/EDTA mixture was defatted by centrifugation at 4500g for approximately 90 minutes at a temperature of +4 °C. The resulting whey fraction was treated with 2 mM and 4 mM Zn^{2+}.

This was applied to a STREAMLINE 50 column (50 mm i.d.) without removing the precipitated material. The column contained 300 ml of STREAMLINE DEAE adsorbent, which corresponds to a sedimented bed height of 15 cm. The flow velocity during expansion/equilibration, adsorption and wash was 300 cm/h. The buffer used during expansion/equilibration and wash was 25 mM Tris/HCl, pH 7.2. Desorption was performed with downward flow in sedimented mode using a three-step elution procedure. The following buffers were used in sequence:

1) 125 mM NaCl in 25 mM Tris/HCl, pH 7.2

2) 250 mM NaCl in 25 mM Tris/HCl, pH 7.2

3) 500 mM NaCl in 25 mM Tris/HCl, pH 7.2

After elution, the bed was regenerated with 2 M NaCl and subjected to a cleaning-in-place (CIP) procedure by washing with a solution of 0.5 M NaOH and 1 M NaCl.

Following the Capture step on STREAMLINE DEAE, the rhPC-containing fraction was further purified by immunoaffinity chromatography. Table 51 summarizes the complete purification process.

Processing whey without addition of Zn^{2+} ions resulted in about 2% of the rhPC and 45% of the total whey proteins passing through the bed without being adsorbed. Treatment with 2 mM Zn^{2+} ions resulted in the elution of 27% of the rhPC and 56% of the total whey proteins in the unbound fraction. The loading of whey treated with 4 mM Zn^{2+} ions resulted in 51% of the rhPC and 85% of the total whey proteins passing through the expanded bed unadsorbed. The 250 mM and 500 mM NaCl step eluates were pooled for subsequent immunoaffinity chromatography. The purification factors of the NaCl step eluates ranged from less than 1 to 8. 29% of the original whey protein and 84% of the rhPC was contained in the pooled eluates from untreated whey loading. The 2 mM Zn^{2+} treated whey loading gave an eluate pool of 23% of the original total whey protein and 66% of the rhPC. The 4 mM Zn^{2+} treated whey loading gave an eluate pool of 18% of the original total whey protein and 41% of the rhPC. The rhPC yield from the immunoaffinity step was 90% to 94% for salt eluate pools and about 86% to 89% for unadsorbed effluent from the STREAMLINE DEAE column. All immunoaffinity products were greater than about 95% pure as judged by SDS-PAGE. A purification factor of about 200 was achieved by combination of STREAMLINE DEAE expanded bed adsorption and immunoaffinity chromatography.

Table 51. Summary of results from the purification of rhPC from milk of transgenic pigs.

Step	No Zinc			2 mM Zinc			4 mM Zinc		
	Total protein (%)	Yield (%)	Purification factor	Total protein (%)	Yield (%)	Purification factor	Total protein (%)	Yield (%)	Purification factor
Feed	100	100	1	100	100	1	100	100	1
Flow-through	44.9	2	0.05	56.2	26.8	0.5	85	51.1	0.6
Elution 1	14.7	4.5	0.3	14.0	1.5	0.1	6.5	0.5	0.1
Elution 2	10.7	83.1	7.8	7.3	50.6	7.0	7	19	2.7
Elution 3	18.1	1.3	0.1	16.0	14.5	0.9	10.7	22.1	2.0
Mab-Affinity Products/ Flow-through	0	0	0	100	86	200	100	89	200
Mab-Affinity Products/ Eluates	100	89	200	100	92	200	100	94	200

Table 52 shows the anticoagulant activity of immunopurified rhPC as percentage of immunopurified hPC derived from human plasma. Immunopurified rhPC from unadsorbed effluents of STREAMLINE DEAE expanded bed adsorption showed no anticoagulant activity by APTT (activated partial thromboplastin time) assay. Essentially, no rhPC was obtained in unadsorbed effluents from loadings of untreated whey. Immunopurified rhPC from NaCl eluate pools of STREAMLINE DEAE showed 43%, 58%, and 75% APTT activity for untreated, 2 mM Zn^{2+} treated, and 4 mM Zn^{2+} treated loadings respectively.

It was concluded that the majority of background proteins and immature populations of rhPC were precipitated and pass through the column unadsorbed in the flow-through fraction when the whey was treated with 4 mM Zn^{2+} prior to loading onto the STREAMLINE DEAE expanded bed. Hence, metal-dependant conformational changes associated with major milk proteins and target protein sub-populations was used as an efficient tool for achieving highly selective expanded bed anion exchange adsorption of transgenic milk.

Table 52. Anticoagulant activity of immunoaffinity purified rhPC STREAMLINE DEAE products.

Metal present	Sample	Purity (%)	Activity by APTT (% hPC ref)
None	STREAMLINE flow-through fraction	NA	NA
	STREAMLINE eluate	>95	43
2 mM Zn^{2+}	STREAMLINE flow-through fraction	>95	0
	STREAMLINE eluate	>95	58
4 mM Zn^{2+}	STREAMLINE flow-through fraction	>95	0
	STREAMLINE eluate	>95	75

8. Fault-finding chart

Start up - Expansion - Equilibration

Problem	Cause	Remedy
Beads stick to column wall when loading the adsorbent.	Adsorbent suspended in water.	Suspend the adsorbent in starting buffer or in a salt solution, e.g. 0.5 - 1.0 M NaCl.
Expanded bed height (H) is lower than expected.	Channelling in the expanded bed due to trapped air in the bottom distribution system.	Try to remove the air by pumping buffer at high flow velocity (e.g. 300-500 cm/h) through the column using downward flow. If the above does not help, remove the adsorbent from the column. Pump distilled water into the column through the bottom distribution system and remove any trapped air using suction from above the adaptor net (see page 53).
	Channelling in the expanded bed due to clogging of the bottom distribution system.	Disassemble the column and clean the distributor plate and net (see column User Manual).
	Channelling in the expanded bed due to the column not being in a vertical position.	Use a spirit level to adjust the vertical position of the column.
	Turbulence in the expanded bed due to fouling, aggregation or infection of the adsorbent.	Clean and/or sanitize the adsorbent (see “Cleaning-in-place”, page 38, and specific cleaning and sanitization recommendations in Section 6, Product Guide).

Problem	Cause	Remedy
Expanded bed height (H) is lower than expected.	Increased density of beads due to fouling.	See above.
	Decreased viscosity due to increased temperature.	Check and control the temperature if necessary.
	Decreased flow velocity due to clogging of the bottom and/or adaptor distribution system.	Disassemble the column and clean the distributor plates and nets (see column User Manual).
	Decreased flow velocity due to folding of the bottom and/or adaptor net.	Replace the net(s).
	Decreased flow velocity due to clogging in valves, connectors, tubing, etc.	Remove and clean the respective parts.
	Decreased flow velocity due to worn pump tubing.	Replace pump tubing. Follow the pump manufacturer´s recommendations for pump use. Most pump manufacturers recommend that the pump tubing is removed from the pump rotor when the pump is not in use to prolong tubing life.
Expanded bed height (H) is higher than expected.	Increased viscosity due to decreased temperature.	Check and control the temperature if necessary.
	Increased flow velocity due to high hydrostatic pressure from buffer tank.	Eliminate (or decrease) the difference in height between the liquid surface in the buffer tank and the system outlet. Alternatively, install a restrictor in the line between the buffer tank and the system inlet.
Low number of theoretical plates (RTD test).	Channelling in the expanded bed due to trapped air in the bottom distribution system.	Try to remove the air by pumping buffer at high flow velocity (e.g. 300-500 cm/h) through the column using downward flow.

Problem	Cause	Remedy
Low number of theoretical plates (RTD test).		If the above does not help, remove the adsorbent from the column. Pump distilled water into the column through the bottom distribution system and remove any trapped air using suction from above the adaptor net (see page 53).
	Channelling in the expanded bed due to clogging of the bottom distribution system.	Disassemble the column and clean the distributor plate and net (see column User Manual).
	Channelling in the expanded bed due to the column not being in a vertical position.	Use a spirit level to adjust the vertical position of the column.
	Turbulence in the expanded bed due to fouling, aggregation or infection of the adsorbent.	Clean and/or sanitize the adsorbent (see "Cleaning-in-place", page 38, and specific cleaning and sanitization recommendations in Section 6, Product Guide).
	Instability in the expanded bed due to low flow velocity giving a low degree of expansion.	Increase flow velocity. Nominal flow velocity is around 300 cm/h at room temperature, giving a degree of expansion of around 3-fold with normal aqueous based buffers.
	Instability in the expanded bed due to unsufficient equilibration.	Extend equilibration time to one hour or more.
	Instability in the expanded bed due to the sedimented bed height being too short.	Increase sedimented bed height. Nominal sedimented bed height is around 15 cm. Minimum recommended sedimented bed height is 10 cm.

Problem	Cause	Remedy
High back pressure.	Clogging of the bottom and/or adaptor distribution system.	Disassemble the column and clean the distributor plates and nets (see column User Manual).
	Folding of the bottom and/or adaptor net.	Replace the net(s).
	Clogging in valves, connectors, tubing etc.	Remove and clean the respective parts.
Fuzzy bed surface.	Presence of adsorbent fines in the expanded bed.	Remove fines by elutriation (see column User Manual).
	Unsufficient equilibration following buffer exchange.	Extend equilibration time after buffer exchange.
Channelling in the lower part of the expanded bed.	Trapped air in the bottom distribution system.	Try to remove the air by pumping buffer at high flow velocity (e.g. 300-500 cm/h) through the column using downward flow.
		If the above does not help, remove the adsorbent from the column. Pump distilled water into the column through the bottom distribution system and remove any trapped air using suction from above the adaptor net (see page 53).
	Clogging of the bottom distribution system.	Disassemble the column and clean the distributor plate and net (see column User Manual).

Problem	Cause	Remedy
Channelling in the lower part of the expanded bed.	Pulsations from the pump.	Change to a pump giving less pulsation, or, install a pulse dampener between the pump and the column, or, change to a smaller pump tubing diameter that will allow the pump to be run at a higher speed.
Large circular movements and liquid channels in the expanded bed.	Column not in a vertical position.	Use a spirit level to adjust the vertical position of the column.
Turbulent flow pattern in the expanded bed.	Fouling, aggregation or infection of the adsorbent.	Clean and/or sanitize the adsorbent (see "Cleaning-in-place", page 38, and specific cleaning and sanitization recommendations in Section 6, Product Guide).

Feed application

Problem	Cause	Remedy
Build up of particulates underneath the adaptor net.	Over-expansion due to high viscosity or high particle content of the feed-stock (high cell density, high biomass content, high content of nucleic acids, low temperature).	Periodically back-flush to clear the adaptor net (see page 56). If build up is frequent, reduce the flow velocity, or, reduce viscosity by diluting feed-stock with buffer or water, or, reduce viscosity by treating the feed-stock with nuclease (e.g. Benzonase) to degrade nucleic acids (see pages 31, 90, 93, 99), or, reduce viscosity by further homogenization of the feed-stock (intracellular products), or, prevent possible release of nucleic acids through cell lysis (extracellular products) by on-line dilution and by increasing osmolality of the diluent (see pages 33, 117, 119). Use fresh cultures to prevent cell lysis (extracellular products) and release of nucleic acids.

Problem	Cause	Remedy
Build up of particulates underneath the adaptor net.	Sedimented bed height is too large.	Decrease sedimented bed height. Nominal sedimented bed height is around 15 cm.
	Aggregation of biomass inside the column is trapped in the adaptor net	Replace the adaptor net with the elutriation sealing.
High back pressure	Build up of particulates underneath the adaptor net.	See above.
	Clogging of the bottom distribution system by nucleic acids in the feed-stock.	Treat the feed-stock with nuclease (e.g. Benzonase) to degrade nucleic acids (see pages 31, 90, 93, 99), and/or, prevent possible release of nucleic acids through cell lysis (extracellular products) by on-line dilution and by increasing osmolality of the diluent (see pages 33, 117, 119), and/or, use fresh cultures to prevent cell lysis (extracellular products) and release of nucleic acids.
	Clogging of the bottom distribution system due to aggregation of biomass in the feed-stock at low pH (e.g. during cation exchange chromatography).	Switch to anion exchange chromatography, which allows a higher pH. Remove large aggregates by an in-line crude mesh filter.
	Clogging of the bottom distribution system due to agglomeration of cells in the feed-stock.	Stir the feed-stock during feed application to prevent cell agglomeration. Remove large agglomerates with an in-line crude mesh filter.

Problem	Cause	Remedy
Reduced flow rate.	High back pressure.	See above.
Clogging of the expanded bed.	Aggregate formation due to interaction of released nucleic acids and/or negatively charged cells with positively charged adsorbent beads (anion exchange adsorbents).	Treat the feed-stock with nuclease (e.g. Benzonase) to degrade nucleic acids (see pages 31, 90, 93, 99), and/or, prevent possible release of nucleic acids through cell lysis (extracellular products) by on-line dilution and by increasing osmolality of the diluent (see pages 33, 117, 119), and/or, use fresh cultures to prevent cell lysis (extracellular products) and release of nucleic acids. If problems are persistent, try a cation exchange adsorbent instead.
Channelling in the expanded bed.	Clogging of the bottom distribution system by nucleic acids in the feed-stock.	Treat the feed-stock with nuclease (e.g. Benzonase) to degrade nucleic acids (see pages 31, 90, 93, 99), and/or, prevent release of nucleic acids through cell lysis (extracellular products) by on-line dilution and by increasing osmolality of the diluent (see pages 33, 117, 119).
	Clogging of the bottom distribution system due to aggregation of biomass in the feed-stock at low pH (e.g. during cation exchange chromatography).	Switch to anion exchange chromatography, which allows a higher pH. Remove large aggregates with an in-line crude mesh filter.

Problem	Cause	Remedy
Channelling in the expanded bed.	Clogging of the bottom distribution system due to agglomeration of cells in the feed-stock.	Stir the feed-stock during feed application to prevent cell agglomeration. Remove large agglomerates with an in-line crude mesh filter.
	Instability caused by high biomass content and high viscosity of the feed-stock.	Reduce viscosity by diluting feed-stock with buffer or water, or, reduce viscosity by treating the feed-stock with nuclease (e.g. Benzonase) to degrade nucleic acids (see pages 31, 90, 93, 99).
Turbulent flow pattern in the expanded bed.	Aggregation, clogging and fouling of the adsorbent due to nucleic acids present in the feed-stock.	Treat the feed-stock with nuclease (e.g. Benzonase) to degrade nucleic acids (see pages 31, 90, 93, 99), If the adsorbent is an anion exchanger, try a cation exchanger instead (if the problems persist).
	Aggregation, clogging and fouling of the adsorbent due to cell lysis (extracellular products), which releases nucleic acids, lipids and other cell membrane components into the feed-stock.	Prevent cell lysis by on-line dilution and by increasing osmolality of the diluent (see pages 33, 117, 119). Use fresh cultures to prevent extensive cell lysis.

Problem	Cause	Remedy
Turbulent flow pattern in the expanded bed.	Aggregation and clogging of the adsorbent due to formation of cell agglomerates and cell/ adsorbent aggregates in the expanded bed.	Stir the feed-stock during feed application to prevent the formation of cell agglomerates, which can enter the bed and form large aggregates of cells and adsorbent beads.
Poor adsorption of target molecule (low recovery or low capacity).	Decreased efficiency of the adsorption process due to channelling and turbulent flow pattern in the expanded bed.	See above.
	Decreased efficiency of the adsorption process due to short residence time.	Increase residence time by decreasing flow rate or by increasing sedimented bed height (see page 43).
	Binding capacity blocked (anion exchange adsorbents) by nucleic acids and other polyanionic macromolecules present in the feed-stock.	Change to a cation exchange adsorbent, or, add Mg^{2+} ions to the feed-stock to form complexes with the negatively charged phosphate groups of the nucleic acids (see page 99).
	Reduced charge on target molecule (cation exchange applications) due to complexing with nucleic acids and other polyanionic macromolecules in the feed-stock.	Change to an anion exchange adsorbent, or, add Mg^{2+} ions to the feed-stock to form complexes with the negatively charged phosphate groups of the nucleic acids.

Problem	Cause	Remedy
Poor adsorption of target molecule (low recovery or low capacity).	Poor binding due to high conductivity in the feed-stock (ion exchange adsorbents).	Dilute the feed-stock to a conductivity below 5 mS/cm, or, minimize conductivity in the culture broth at the end of the fermentation process, or, try the high capacity ion exchangers STREAMLINE Q XL or STREAMLINE SP XL.

Wash

Problem	Cause	Remedy
Increased wash volume/time.	Channelling in the expanded bed due to aggregation caused by nucleic acids being released during lysis of cells retarded on the adsorbent beads.	Apply a wash procedure containing nuclease (e.g. Benzonase) to degrade and remove nucleic acids from the bed (see page 36).

Elution

Problem	Cause	Remedy
High back pressure.	Formation of large aggregates during application of feed-stock that are not removed during the washing stage.	Elute in expanded bed mode (see page 37). Remove the aggregates during cleaning-in-place.
Large product volume.	Excessive zone spreading during elution.	Decrease flow velocity during elution.
		Increase elution strength of the eluting buffer.
		Change flow direction.
		Pause the system (stop the flow) after having filled the bed with eluent buffer. Continue with elution after a static incubation time of approximately one hour.
Precipitation in the eluate	Elution by low pH causing precipitation of nucleic acids present in the bed at the start of elution.	Apply a wash procedure containing nuclease (e.g. Benzonase) to degrade and remove nucleic acids from the bed prior to elution (see page 36).
Low yield	Degradation of target molecule by proteases released upon cell lysis (extracellular products).	Prevent cell lysis by on-line dilution and by increasing the osmolality of the diluent (see pages 33, 117, 119).
		Use fresh cultures to prevent extensive cell lysis.

9. Ordering information

Adsorbents

Product	Pack size	Code No.
STREAMLINE SP	100 ml	17-0993-05
	300 ml	17-0993-01
	7.5 L	17-0993-02
	60 L	17-0993-03
STREAMLINE DEAE	100 ml	17-0994-05
	300 ml	17-0994-01
	7.5 L	17-0994-02
	60 L	17-0994-03
STREAMLINE SP XL	100 ml	17-5076-05
	300 ml	17-5076-01
	7.5 L	17-5076-02
STREAMLINE Q XL	100 ml	17-5075-05
	300 ml	17-5075-01
	7.5 L	17-5075-02
STREAMLINE Chelating	300 ml	17-1280-01
	7.5 L	17-1280-02
STREAMLINE Heparin	75 ml	17-1284-06
	300 ml	17-1284-01
	7.5 L	17-1284-02
STREAMLINE rProtein A	75 ml	17-1281-01
	300 ml	17-1281-02
	5 L	17-1281-03

Columns

Product	Code No.
XK 16/20 (16 mm i.d.)	18-8773-01
XK 26/20 (26 mm i.d.)	18-1000-72
STREAMLINE 25 (25 mm i.d.) equipped with hydraulic adaptor	18-1110-50
STREAMLINE 25 (25 mm i.d.) equipped with manual adaptor	18-1110-51
STREAMLINE 50 (50 mm i.d.) equipped with hydraulic adaptor	18-1038-01
STREAMLINE 200[1] (200 mm i.d.) equipped with hydraulic adaptor	18-1100-22
[1] Stand (must be ordered separately)	18-1031-20
STREAMLINE CD	For ordering, contact your nearest Pharmacia Biotech office

Systems

Product	Code No.
STREAMLINE manual (portable) system; 6 mm i.d.	44-9431-01
STREAMLINE manual (portable) system; 10 mm i.d.	44-9431-03
STREAMLINE pilot scale systems	For ordering, contact your nearest
STREAMLINE production scale systems	Pharmacia Biotech office

Other components

Item	Qty/pack	Code No.
Valves		
SRV-3	1	19-2098-01
SRV-4	1	19-2099-01
L-type; 1/4”; PP	1	19-0239-01
L-type; 6 mm; SS	1	18-5757-01
L-type; 1/2”; SS	1	18-1001-37
L-type; 10 mm; SS	1	18-1012-56
4-way; 1/4”; PP	1	19-0240-01
4-way; 6 mm; SS	1	18-5758-01
4-way; 1/2”; SS	1	18-1001-36
4-way; 10 mm; SS	1	18-1012-57
Solenoid valve PSV-50	1	19-1994-01
Connectors		
M6; 2.7 mm	5	18-4652-01
JACO 10-4-2; 1/4”	3	19-0273-01
JACO 10-8-6; 1/2”	5	18-6880-01
SRTC-3	2	19-2144-01
Unions		
25 mm o.d. clamp to M6	2	18-1031-09
25 mm o.d. clamp to 1/4” threaded	2	18-0251-98
25 mm o.d. clamp to 1/2” threaded	2	18-1012-68
Tubing		
1.9 x 2.7 mm; PTFE; unflanged	2 x 1 m	18-8210-01
	1 x 2 m	18-8207-01
	1 x 25 m	18-8207-02
1.9 x 2.7 mm; PTFE; S-flanged	1 x 1 m	18-8207-03
1/4”; PE	1 x 5 m	19-0385-01
1/2”; PE	1 x 5 m	18-1015-10
6 mm; PVC	1 x 30 cm	18-0005-42
	1 x 75 cm	18-0005-43
	1 x 125 cm	18-0005-44
	1 x 150 cm	18-0005-45
	1 x 200 cm	18-0005-47
10 mm; PVC	1 x 30 cm	18-1012-85
	1 x 40 cm	18-1012-86
	1 x 90 cm	18-1012-62
	1 x 140 cm	18-1012-63
	1 x 170 cm	18-1012-64
	1 x 200 cm	18-1012-87

Peristaltic tubing		
1.6 mm i.d.	Contact your nearest Pharmacia Biotech office for ordering. Peristaltic tubing can be supplied with moulded-on 25 mm o.d. clamp connectors on request.	
3.2 mm i.d.		
9.6 mm i.d.		
Pumps		
HiLoad Pump P-50	1	19-1992-01
Watson Marlow 504 U/RL (peristaltic)	1	44-2677-05
Watson Marlow 604 U/R (peristaltic)	1	44-2686-01
UV-monitor/Flow cells		
Monitor UV-1	1	18-1003-66
S-2 flow cell	1	19-4840-02
Industrial flow cell; 6 mm i.d.	1	18-1000-66
Industrial flow cell; 10 mm i.d.	1	18-1000-65
280 nm Filter kit	1	19-2433-01
Miscellaneous		
GradiFrac (includes 2 solenoid valves PSV-50)	1	18-1993-01
GradiFrac Rack	1	18-1993-05
Recorder REC 101 (single-channel operation)	1	18-1001-42
Recorder REC 102 (dual-channel operation)	1	18-1001-43
Plastic clamp; 25 mm o.d.	5	44-0508-05
Gasket; 6 mm i.d.	100	44-0581-01
Gasket; 10 mm i.d.	100	44-0581-02
Blind flange (25 mm o.d.) and packing	1	18-1001-25
Stop plug	2	19-5170-01
Flanging kit[1] 120 V	1	18-4603-70
Flanging kit[1] 220 V	1	18-4603-71

[1] To flange tubing (1.9 x 2.7 mm) ends so that the tubing is retained inside the tubing connector (M6)

10. References

1. *Methods of Plasma Protein Fractionation.* Curling, J.M., Ed.; Academic Press: London, UK, *1980, pp. 117-128,* Brummelhuis, H.G.J.
2. A novel ion-exchange method for the isolation of streptomycin. *Chem. Eng. Prog. 54 (1958) 49-52,* Barthels, C.R., Kleinman, G., Korzon, N.J., Irish, D.B.
3. Development of a recovery process for novobiocin. *Biotechnol. Bioeng. 15 (1973) 533-549,* Belter, P.A., Cunningham, F.L., Chen, J.W.
4. Fluidized bed adsorption for whole broth extraction. *Biotechnol. Prog. 6 (1990) 370-375,* Gailliot, F.P., Gleason, C., Wilson, J.J., Zwarick, J.
5. Batch fluidized ion-exchange column for streams containing suspended particles. *J. Chromatogr. 201 (1980) 319-327,* Buijs, A., Wesselingh, J.A.
6. Continuous affinity chromatography using a magnetically stabilized fluidized bed. *Biotechnol. Prog. 1 (1985) 95-103,* Burns, M.A., Graves, D.J.
7. The effects of magnetic stabilization on the structure and performance of fluidized beds. *Bioseparations 2 (1991) 217-230,* Nixon, L., Koval, C.A., Xu, L., Noble, R.D., Slaff, G.S.
8. Liquid fluidized beds for protein purification. *I. Chem. Eng. Symp. Ser. No. 118: 12.1-12.12 (1990),* Draeger, M.N., Chase, H.A.
9. Liquid fluidized bed adsorption of proteins in the presence of cells. *Bioseparations 2 (1991) 67-80,* Draeger, M.N., Chase, H.A.
10. Affinity purification of proteins using expanded beds. *J. Chromatogr. 597 (1992) 129-145,* Chase, H.A., Draeger, M.N.
11. Expanded bed adsorption of proteins using ion-exchangers. *Separation Sci. Technol. 27 (1992) 2021-2039,* Chase, H.A., Draeger, M.N.
12. Adapting Chromatography for Initial Large-Scale Protein Recovery. ACS Conference Proceedings Series, *Harnessing Biotechnology for the 21st Century. Ladisch, M., Bose, A., Eds.; 1992, pp. 271-274,* Hedman, P., Barnfield Frej, A.-K.
13. Characterization of the Internal Flow Hydrodynamics in an Expanded Bed Adsorption Column. Poster presented at *Recovery of Biological Products VI. Engineering Foundation, Interlaken, Switzerland, September 1992,* Wnukowski, P., Lindgren, A.
14. Recovery of a Recombinant Protein from an *E. coli* Homogenate using Expanded Bed Adsorption. Poster presented at *Recovery of Biological Products VI. Engineering Foundation, Interlaken, Switzerland, September 1992,* Barnfield Frej, A.-K., Johansson, S., Hjorth, R.

15. Hydrodynamic Stability of the Liquid Fluidized Bed of Small Particles: An Experimental Study. Poster presented at *AIChE Annual Meeting, Florida, USA, November 1992, Paper no. 116dd,* Johansson, B.U., Wnukowski, P.

16. High-performance liquid chromatography of amino acids, peptides and proteins. CXXIV. Physical characterization of fluidized-bed behaviour of chromatographic packing materials. *J. Chromatogr. 631 (1993) 115-124,* Dasari, G., Prince, I., Hearn, M.T.W.

17. Expanded Bed Adsorption. The first new unit process operation in decades. *Bio/Technol. 11 (1993) 1059,* McCormick, D.K.

18. Expanded Bed Adsorption - A New Way for Industrial Recovery of Recombinant Proteins. Poster presented at *New Zealand Biotech Association Meeting, Palmerston North, New Zealand, May 1993,* Schmidt, C. et al.

19. Characterization of a Novel Adsorbent for Recovery of Proteins in Expanded Beds. Poster presented at *6th European Congress on Biotechnology, Florence, Italy, June 1993,* Kämpe, S., Hjorth, R., Nyström, L.-E.

20. Expanded Purification of Proteins using Purpose Designed Adsorbents. *6th European Congress on Biotechnology, Florence, Italy, June 1993, Volume III, p. WE 013,* Chase, H.A., Chang, Y.-K..

21. Purification of Proteins from Crude Feedstock using STREAMLINE Expanded Bed Adsorption. Presented at *Thirteenth International Symposium on HPLC of Proteins, Peptides and Polynucleotides, San Francisco, USA, November 30-December 3, 1993,* Kämpe, S., Barnfield Frej, A.-K. et al.

22. Analysis of some operating parameters of Novel Adsorbents for Recovery of Proteins in Expanded Beds. *Bioseparation 5 (1995) 217-223,* Hjorth, R., Kämpe, S., Carlsson, M.

23. Development of operating conditions for protein purification using expanded bed techniques: The effect of the degree of bed expansion on adsorption performance. *Biotech. & Bioeng. 49 (1996) 512-526,* Chang, Y.-K., Chase, H.A.

24. Protein Recovery from *E. coli* Homogenate using Expanded Bed Adsorption Chromatography. Presented at *205th American Chemical Society National Meeting, Denver, Colorado, USA, April 1993, Paper no. 61,* Suding, A., Tomusiak, M.

25. Expanded Bed Adsorption Process for Protein Recovery from Whole Mammalian Cell Culture Broth. *Bioseparations 5 (1995) 41-52,* Batt, B.C., Yabannavar, V.M., Singh, V.

26. Impact of Improved Chromatographic Media on Productivity and Process Design in Downstream Processing. Poster presented at *12th Meeting of European Animal Cell Technology, Würtzburg, Germany, May 1993,* Schmidt, C., Berglöf, J. H., Lindquist, L.-O.

27. Pilot Scale Purification of Recombinant Annexin V using Expanded Bed Adsorption, STREAMLINE, and Hydrophobic Interaction Chromatography, Butyl Sepharose 4 Fast Flow. Poster presented at *6th European Congress on Biotechnology, Florence, Italy, June 1993,* Sköld, A., Daniels, I., Barnfield Frej, A.-K.

28. Pilot Scale Recovery of Recombinant Annexin V from Unclarified *Escherichia coli* Homogenate using Expanded Bed Adsorption. *Biotech. & Bioeng. 44 (1994) 922-929,* Barnfield Frej A.-K., Hjorth, R., Hammarström, Å.

29. Purification of Recombinant Anti-HIV Fab-Fragment Expressed in *Escherichia coli*. Poster presented at *Recovery of Biological Products VII in San Diego, California, USA, September 1994,* Jägersten, C. et al.

30. Purification of a Recombinant Bacterial Exotoxin A by Expanded Bed Adsorption and a New Ion Exchange Media - SOURCE 30Q. Poster presented at *Prep Tech ´95, Rutherford, New Jersey, USA, 1995,* Johansson, H.J., Shiloach, J., Jägersten, C.

31. STREAMLINE Expanded Bed Adsorption for Recovery of Renatured Human Interleukin 8 from *Escherichia coli. Bioseparations (1997), In press,* Barnfield Frej, A.-K., Hammarström, Å., Jones, I., Hjorth, R.

32. Single-step Recovery of a Secreted Recombinant Protein by Expanded Bed Adsorption. *Bio/Technol. 12 (1994) 285-288,* Hansson, M., Ståhl, S., Hjorth, R., Uhlén, M., Moks, T.

33. Development of an Expanded Bed Technique for an Affinity Purification of G6PDH from Unclarified Yeast Cell Homogenates. *Biotech. & Bioeng. 48 (1995) 355-366,* Chang, Y.-K., McCreath, G.E., Chase, H.A.

34. Ion Exchange Purification of G6PDH from Unclarified Yeast Cell Homogenates using Expanded Bed Adsorption. *Biotech. & Bioeng. 49 (1996) 204-216,* Chang, Y.-K., Chase, H.A.

35. Purification of Monoclonal Antibodies from Whole Hybridoma Fermentation Broth by Fluidized Bed Adsorption. *Biotech. & Bioeng. 45 (1995) 205-211,* Thömmes, J. et al.

36. Direct Capture of Recombinant Proteins from Animal Cell Culture Media using a Fluidized Bed Adsorber. *Animal Cell Technology: Products for today, prospects for tomorrow.* Griffiths, B., Spier, R. E., Berthold, W., Eds.; Butterworth & Heinemann, Oxford, *1994, pp. 557-560,* Erickson, J.C., Finch, J.D., Greene, D.C.

37. Scale-up of Expanded Bed Adsorption Processes. Poster presented at *7th BioProcess Engineering Symposium, Book No. H00887 - 1993,* Lindgren, A., Johansson, S., Nyström, L.-E.

38. Scale-up Validation of Expanded Bed Adsorption Processes. Poster presented at *6th European Congress on Biotechnology, Florence, Italy, June 1993,* Johansson, S., Lindgren, A., Nyström, L.-E.

39. Expanded bed adsorption at production scale: Scale-up verification, process example and sanitization of column and adsorbent. *Bioprocess Engineering 16 (1997) 57-63,* Barnfield Frej, A.-K., Johansson, H.J., Johansson, S., Leijon, P.

40. Purification of Recombinant Human Retinoblastoma Protein at Canji Inc. using STREAMLINE Expanded Bed Adsorption. *Downstream No. 17 (1994),* Pharmacia Biotech AB.

41. *European Patent Application EP 0 699 687 A2 (1996).* Noda, M., Sumi, A., Ohmura, T., Yokoyama, K.

42. Purification of Proteins by Adsorption Chromatography in Expanded Beds. *Trends in Biotech. 12 (1994) 296-303,* Chase, H.A.

43. Physical-Chemical Properties of STREAMLINE Ion Exchangers. Poster presented at *7th European Congress on Biotechnology, Nice, France, February 1995,* Hansson, K.-A.

44. Expanded Bed Adsorption: Optimization of the Wash and Elution Steps on STREAMLINE Ion Exchangers. Poster presented at *7th European Congress on Biotechnology, Nice, France, February 1995,* Carlsson, M.

45. STREAMLINE SP/STREAMLINE DEAE Cleaning-in-place. *Application Note 18-1115-27,* Pharmacia Biotech AB.

46. Cleaning In Place (CIP) of STREAMLINE rProtein A. Poster presented at *Cell Culture Engineering V, San Diego, California, USA, January 28 - February 2, 1996,* Asplund, M., Kämpe, S., Jägersten, C.

47. Cleaning, Sanitization and Storage. *Handbook of Process Chromatography. A Guide to Optimization, Scale up, and Validation, Academic Press, London, UK, ISBN: 0-12-654266,* Hagel, L., Sofer, G.

48. Metal Chelate Affinity Chromatography, a new approach to protein purification. *Nature 258 (1975) 598-599,* Porath, J., Carlsson, J., Olsson, I. et al.

49. Purification of Proteins by IMAC. *Trends in Biotechnol. 3 (1985) 1-7,* Sulkowski, E.

50. Protein Interactions with Immobilized Transition Metal Ions: Quantitative evaluations of variations in affinity and binding capacity. *Anal. Biochem. 191 (1990) 160-168,* Hutchens, T.W., Yip, T.-T.

51. Proteoglycans: structures and interaction. *Ann. Rev. Biochem. 60 (1991) 443-475,* Kjellén, L., Lindahl, U.

52. STREAMLINE 25 column. *Data File 18-1112-02,* Pharmacia Biotech AB.

53. Large scale recovery and purification of periplasmic recombinant protein from *E. coli* using expanded bed adsorption chromatography followed by new ion exchange media. *J. Biotechnol. 48 (1996) 9-14,* Johansson, H.J., Jägersten, C., Shiloach, J.

54. Purification of Secreted Recombinant Proteins from Hansenula polymorpha by Fluidized Bed Adsorption. Poster presented at *First International Conference on Expanded Bed Adsorption (EBA ´96), Cambridge, UK, December 1996, Paper no. P6.5,* Gellissen, G., Keup, P., Thömmes, J., Kula, M.-R.

55. Simple two-step procedure for the preparation of highly active pure equine milk lysozyme. *J. Chromatogr. 719(1996) 327-331,* Noppe, W., Hanssens, I., De Cuyper, M.

56. Production of Two Aprotinin Variants in *Hansenula polymorpha. Process Biochemistry 31 (1996) 679-689,* Zurek, C., Kubis, E., Keup, P., Hörlein, D., Beunink, J., Thömmes, J., Kula, M.-R., Hollenberg, C.P., Gellissen, G.

57. A General Method for the Purification of Recombinant Proteins Expressed in *E. coli.* Poster presented at *Prep ´96, Washington, USA, May 1996,* Daniels, A.I., Björk, P., Ljunglöf, A. Danielsson, Å.

58. Purification of a therapeutic recombinant protein using expanded bed adsorption chromatography. *Downstream No. 23 (1996),* Pharmacia Biotech AB. Ollivier, M., Bussone, P., Wallet, J.C.

59. Purity of Recombinant Protein A, Used as a Ligand in New Affinity Chromatography Media - Validation of Analytical Methods. Poster presented at *Pittcon ´96, Chicago, USA,* Isaksson, K., Hellberg, U., Moberg, A.

60. Expanded Bed Purification of a Recombinant Protein from the Milk of Transgenic Livestock. Presented at *211th American Chemical Society National Meeting, New Orleans, Louisiana, USA, March 24-28, 1996,* Degener, A., Belew, M., Velander, W.H.

61. (Contract) Manufacture of Clinical Grade (CHO-Recombinant Human) Mab´s: Perfused Fluidized Bed Production, Expanded Bed Affinity Purification and Validation. Poster presented at *Cell Culture Engineering V, San Diego, California, USA, January 28 - February 2, 1996,* Katinger, H., Schmatz, C., Lenz, S., Koller, G., Kreismayr, G., Klima, G., Unterluggauer, F., Katinger, D., Doblhoff-Dier, O.

62. Construction of a rProtein A Affinity Media for use in Expanded Bed Adsorption Chromatography. Presented at *211th American Chemical Society National Meeting, New Orleans, Louisiana, USA, March 24-28, 1996,* Kennedy, R.M.

63. Large-Scale Purification and Characterization of Recombinant Fibroblast Growth Factor-Saporin Mitotoxin. *Protein Expression and Purification 8 (1996) 97-108,* McDonald, J.R., Ong, M., Shen, C., Parandoosh, Z., Sosnowski, B., Bussel, S., Houston, L.L.

64. Isolation of monoclonal antibodies from cell containing hybridoma broth using a protein A coated adsorbent in expanded beds. *J. Chromatogr. A 752 (1996) 111-122,* Thömmes, J., Bader, A., Halfar, M., Karau, A., Kula, M.-R.

65. Capture of a Humanized IgG4 Directly from the Fermenter Using STREAMLINE rProtein A. Presented at *Recovery of Biological Products VIII, Tucson, Arizona, October 20-25, 1996, Abstr. p. 16,* Jägersten, C., Johansson, S., Bonnerjea, J., Pardon, R.

66. Expanded Bed Adsorption Chromatography Purification of a Monoclonal Antibody. Presented at *Recovery of Biological Products VIII, Tucson, Arizona,October 20-25, 1996, Abstr. p. 16,* Zapata, G., Lindgren, A., Barnfield Frej, A.-K., Leijon, P., Liten, A., Mayes, T. L., et al.

67. Direct Capture of Nerve Growth Factor from CHO Cell Culture by EBA. Presented at *Recovery of Biological Products VIII, Tucson, Arizona, October 20-25, 1996, Abstr. p. 74,* Beck, J., Liten, A., Viswanathan, S., Emery, C., Builder, S.

68. A Comparative Engineering Study of the Use of Expanded Bed and Packed Bed Routes for the Recovery of Labile Proteins from Crude Feedstocks. Proceeding: *5th World Congress of Chemical Engineering, July 1996, Vol. 2, 565-570,* Smith, M.P., Bulmer, M., Hjorth, R., Titchener-Hooker, N.J.

69. STREAMLINE Chelating: Characterization of a New Adsorbent for Expanded Bed Adsorption. Poster presented at *First International Conference on Expanded Bed Adsorption (EBA ´96), Cambridge, UK, December 1996, Paper no. P5.7,* Blomqvist, I., Lagerlund, I., Larsson, L.-J., Westergren, H., Shiloach, J.

70. An approach to integrated antibody production: Coupling of fluidized bed cultivation and fluidized bed adsorption. *Bioprocess Engineering 15 (1996) 21-29,* Born, C., Thömmes, J., Biselli, M., Wandrey, C., Kula, M.-R.

71. Polymer-Shielded Dye-Ligand Chromatography of Lactate Dehydrogenase from Porcine Muscle in an Expanded Bed System. *Bioseparations 6 (1996) 193-199,* Garg, N., Yu, I., Mattiasson, G., Mattiasson, B.

72. Purification of Human Recombinant Interleukin 1 Receptor Antagonist Proteins upon *Bacillus subtilis* Sporulation. *Protein Expression and Purification 9 (1997) 219-227,* Maurizi, G., Di Cioccio, V., Macchia, G., Bossù, P., Bizzarri, C., Visconti, U., Boraschi, D., Tagliabue, A., Ruggiero, P.

73. Direct Capture of Monoclonal Antibodies using a New rProtein A Matrix in Fluidized Bed Chromatography under Lysis Free Conditions. Poster presented at *Recovery of Biological Products VIII, Tucson, Arizona, October 20-25, 1996,* Lütkemeyer, D., Ameskamp, N., Tebbe, H., Wittler, J., Lehmann, J.

74. Development of Immobilised Metal Affinity Chromatography of Proteins in Expanded Beds. Poster presented at *First International Conference on Expanded Bed Adsorption (EBA ´96), Cambridge, UK, December 1996, Paper no. P5.8,* Clemmit, R.H., Ghose, S., Chase, H.A.

75a. The recovery of a Recombinant Therapeutic Protein from a High Cell Density Fermentation Process Using Expanded Bed Adsorption Chromatography. Poster presented at *First International Conference on Expanded Bed Adsorption (EBA '96), Cambridge, UK, December 1996, Paper no. P6.3,* Binieda, A., Lewis, C.A., Pearce-Higgins, M., Purvis, J.A.

75b. Expanded Bed Adsorption Chromatography: A Case Study from the Real World. Poster presented at *First International Conference on Expanded Bed Adsorption (EBA '96), Cambridge, UK, December 1996, Paper no. P6.4,* Purvis, J.A., Binieda, A., Lewis, C.A., Pearce-Higgins, M., Varley, P.G.

76. Protein Separation from Transgenic Milk. *J. Chem. Tech. Biotechnol. 54 (1994) 110,* Wright, G., Binieda, A., Udell, M.

77. A scalable method for the purification of recombinant human protein C from the milk of transgenic swine. *Adv. Bioproc. Eng. (1994) 501-507,* Drohan, W.N., Wilkins, T.D., Latimer, E., Zhou, D., Velander, W., Lee, T.K., Lubon, H.

78. Estimating plate-heights in stacked membrane chromatography by flow reversal. *J. Chromatogr. A 702 (1995) 69-80*, Roper, D.K., Lightfoot, E.N.

79. Review of liquid mixing in packed bed biological reactors. *Biotechnol. Progr. 4 (1988) 134-148*, Swaine, D.E., Daugulis, A.J.

80. Analysis of boundary conditions in the axial dispersion model by application of numerical laplace inversion. *Chem. Eng. Sci. 46 (1991) 2567-2571,* Seidel-Morgenstern, A.

81. *Chemical Reaction Engineering, Wiley & Sons, New York (1977),* Levenspiel, O.

APPENDIX

AXIAL MIXING IN LIQUID FLUIDIZED BEDS

The main difference between adsorption in packed beds and in fluidized beds is the mobility of the adsorbent particles within the fluidized bed. Therefore, the traditional limiting factors of protein adsorption to porous matrices have to be extended by mixing in the solid phase, which arises from particle movement during fluidization. These limiting factors are equilibrium/kinetics of the protein/ligand interaction, mixing in the liquid phase, fluid side transport and particle side transport. Up to now, no data are available on particle mixing in beds of fluidized adsorbents. However, it can be suspected that the particle movement will also lead to increased fluid phase mixing. A short theoretical description of mixing phenomena is presented below. Some methods of correct quantification of mixing in liquid fluidized beds are also explained.

In general, the expression axial mixing summarises all possible deviations from a plug flow movement of fluid elements in a chromatographic bed, irrespective whether it is fluidized or packed. In a more precise picture, three different contributions to overall mixing are found, as has been pointed out by Roper and Lightfoot for adsorptive membranes (78). Their considerations are also valid in this case.

Firstly, micro scale fluid phase mixing, which may be caused by mechanical obstructions to regular flow (recirculation, eddies etc.), as well as by diffusion, comprises the so-called convective dispersion.

A second contribution is the presence of different flow paths through the adsorbent bed, which results in a broad distribution of residence times of fluid elements. In the case of liquid fluidized beds, channels at the column inlet or zones of preferential passage through the bed are examples of these phenomena.

The third effect comes from extra column mixing, e.g. in large tubing volumes, mixing plates at column inlets, or in detectors. These effects are very important in short adsorbent beds as the ratio of extra column residence time to the residence time in the bed is unfavourable.

To get an overall picture of mixing by simple means, these contributions are usually not isolated in fluidized bed adsorption. The result without these considerations will still be sufficient to judge whether a fluidized bed is stabilized so that it will allow efficient adsorption. However, it may be helpful to consider these different sources of mixing when trouble-shooting cases of increased mixing within the adsorbent bed.

The simplest way of describing the overall mixing in a liquid fluidized bed is via the residence time distribution (RTD) of fluid elements. RTD describes the probability distribution of a fluid element spending a certain time t in the column. A wide RTD represents a situation where gross mixing of liquid elements leads to a broad range

of possible times t that a fluid element can spend in the column. If we consider the application to a column of an infinitely narrow pulse of a suitable tracer that does not interact with the adsorbent, ideal plug flow would demand that the pulse travels unchanged through the bed, so that each tracer molecule has precisely the same residence time t'. This residence time is calculated from the ratio of bed length to flow velocity (L/v). If one or more of the factors discussed above causes axial mixing, then some elements of the pulse will be retarded and have increased t'. Other parts will travel faster and show reduced t'. Thus, a variety of t' is obtained, which is characterised by the residence time distribution function E(t).

There are two simple models that can mathematically describe RTD as a function of distinct parameters and quantify the amount of axial mixing within a column; the dispersion model and the tanks in series model.

The dispersion model starts with a mass balance over a thin segment of an adsorbent column. Considering convective and dispersive transport results in the well-known equation (1)

(1)
$$\frac{\partial c}{\partial t} = D_{ax} \cdot \frac{\partial^2 c}{\partial z^2} - v \cdot \frac{\partial c}{\partial z}$$

Under the boundary conditions of an open system, an analytical solution may be obtained (eq. 2), which describes E(t) as a function of the dimensionless time Θ ($=t/\tau$) and of a single variable, the column Peclet number *Pe*, which is defined according to equation (3) (79).

(2)
$$E(\Theta) = \frac{Pe}{\sqrt{4 \cdot \pi \cdot \Theta}} \cdot \exp\left(- \frac{Pe \cdot (1-\Theta)^2}{4 \cdot \Theta} \right)$$

(3)
$$Pe = \frac{v \cdot L}{D_{ax}}$$

Dax is the overall
coefficient of axial mixing (m^2/s). *Pe* is a dimensionless number that relates dispersed flow (D_{ax}) to convective flow ($v \cdot L$). Increasing *Pe* then stands for decreased axial mixing. As may be seen from the definition of *Pe*, increasing bed length or flow velocity reduces the overall mixing within an adsorbent bed. This dimensionless group has also been called Bodenstein number *Bo*. A third dimensionless number representing axial mixing is the particle Peclet number Pe_p, that contains the particle diameter d_p as the characteristic length. It therefore characterises a specific adsorbent particle rather than the specific column set-up.

(4)
$$Pe_p = \frac{v \cdot d_p}{D_{ax} \cdot \varepsilon}$$

If the boundary conditions of a closed system are chosen, then an analytical solution of eq. (1) cannot be obtained and the equation has to be solved numerically (80). From eq. (1), the broadening of a tracer pulse due to axial mixing may be estimated. Figure 42 shows a set of curves for different degrees of mixing (represented by differing Bodenstein number *Bo*). The ideal plug flow would be characterised by infinitely high *Bo*.

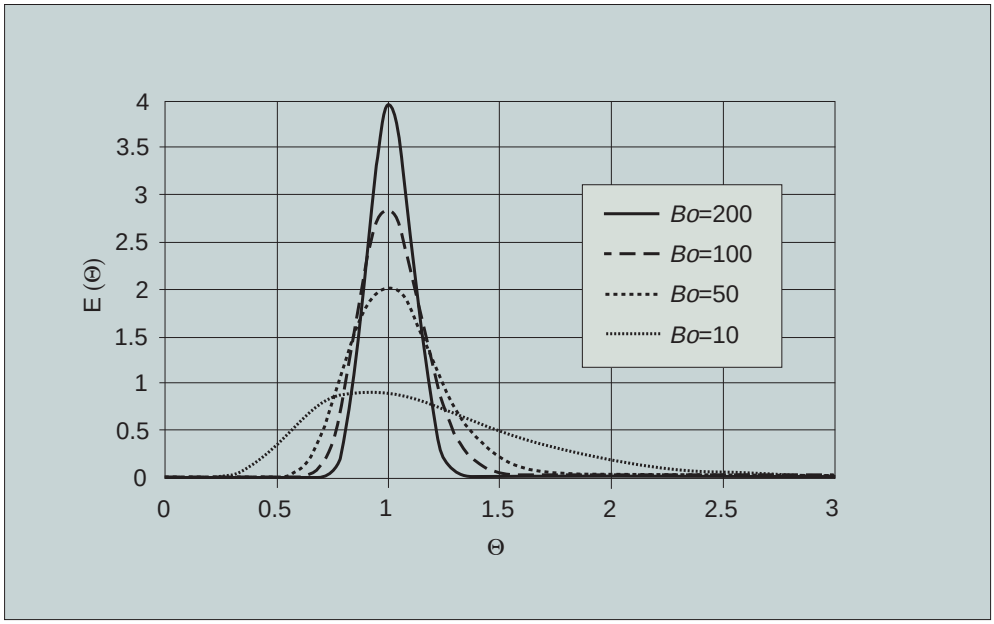


Fig. 42. A set of RTD curves for different degrees of axial mixing (represented by differing Bodenstein number *Bo*)

The tanks in series model considers the column to exist from a cascade of ideal stirred tank reactors. The larger the number of tanks in the cascade, the closer the mixing behaviour is to ideal plug flow. For the tanks in series model, an analytical solution may also be found (equation 5) describing E(Θ) as a function of the number of tanks *N*.

(5)

$$E(\Theta) = \frac{N \cdot (N \cdot \Theta)^{N-1}}{(N-1)!} \cdot \exp(-N \cdot \Theta)$$

Figure 43 presents a series of RTD curves for different values of *N*. Again, an infinitely high tank number represents ideal plug flow.

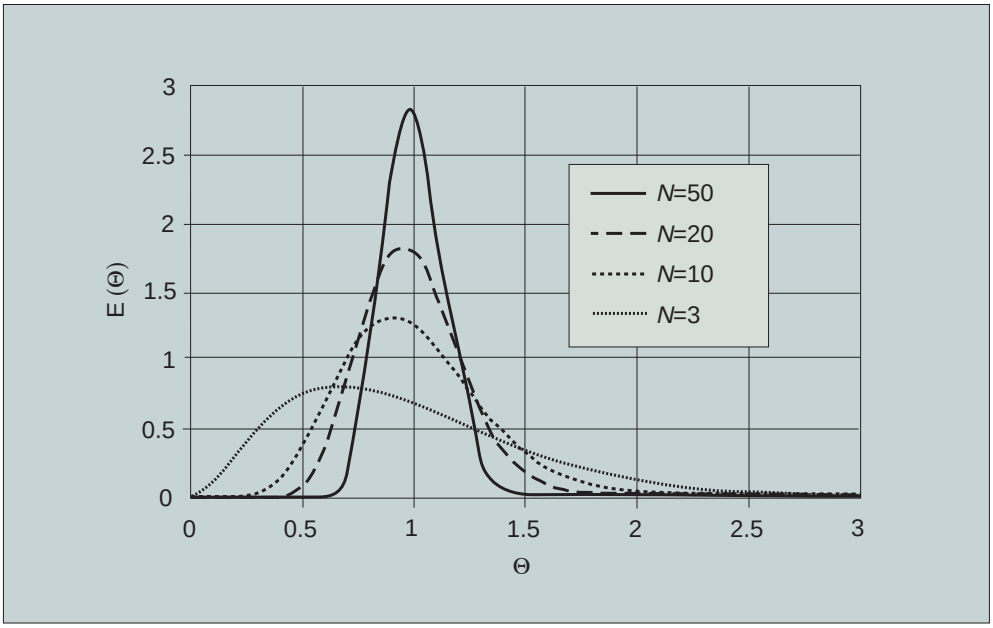


Fig. 43. A set of RTD curves for different degrees of axial mixing (represented by differing values of *N*.

The dimensionless groups *Pe* and *Bo* as well as *N* may be determined by fitting the analytical solutions according to equations (2) and (5) to experimentally obtained RTD curves by non-linear regression. The coefficient of axial mixing *Dax* is obtained from the dispersion model. Applying the tanks in series model yields *N*, which is similar to the number of stages according to the HETP concept under non-binding conditions. Both models allow quantification of axial mixing in liquid fluidized beds from tracer pulse experiments.

A distribution curve like the RTD may be quantified by the moments of the distribution, which are calculated according to equation 6.

(6)

$$m_k = \int_0^\infty t^k \cdot C(t)dt$$

C(t) is the concentration at the column outlet, m_0 stands for the area below the concentration-time curve (the total amount of tracer applied) and is used to normalize the moments. The first normalized moment μ_1 represents the mean residence time τ, the second normalised moment μ_2 is used to calculate the variance of the distribution σ^2, as shown in equations 7 and 8.

(7)

$$\mu_1 = \tau = \frac{\int_0^\infty t \cdot C(t)dt}{\int_0^\infty C(t)dt}$$

(8)

$$\sigma^2 = \mu_2 - \mu_1^2$$

Mean and variance of the RTD may now be used to calculate the dimensionless groups *Bo* or *Pe* as well as *N* without needing to perform non-linear regression (81).

Under the boundary conditions of a closed system, σ^2 is correlated to *Bo* (*Pe*) according to equation (9). Equation (10) is used to calculate the tank number *N*.

(9)

$$\sigma_\Theta^2 = \frac{2}{Bo} \cdot \left(1 - \frac{1}{Bo} \cdot \left(1 - \exp(-Bo)\right)\right)$$

(10)

$$\sigma_\Theta^2 = \frac{1}{N}$$

If a correct experimental set up is chosen (especially with regard to selecting a tracer and applying the pulse correctly), then the methods described above can be used to describe overall mixing in a liquid fluidised bed to decide whether the bed will provide efficient adsorption from a dispersion point of view, or whether bed stability has to be improved prior to the adsorption process. As a general rule of thumb, $Bo > 40$ or $N > 20$ may be regarded as sufficient to ensure that the adsorption process is not limited by liquid mixing.

Trademarks

The following designations are trademarks owned by Pharmacia Biotech AB: STREAMLINE, EXTREME LOAD, FineLINE, Sephadex, Sepharose, Mono Q, SOURCE, GradiFrac, ÄKTA, BioPilot, BioProcess, UNICORN.

Order from

Head Office

Pharmacia Biotech AB
Björkgatan 30
S-751 82 Uppsala, Sweden
Tel: +46 (0) 18 16 50 00
Fax: +46 (0) 18 16 64 05

North America

USA T: 1-800-526-3593
between 8:30 am and 8:00 pm EST
F: 1-800-FAX-3593

Canada T: 1-800-463-5800
between 8:30 am and 6:00 pm EST
F: 1-800-567-1008

Central & South America

Brazil T: +55 11 872 6833
F: +55 11 873 0464
E-mail: pharmaciabrasil@originet.com.br

Europe

Main European office (Freiburg, Germany)
T: +49 (0) 761 4519 0 F: +49 (0) 761 4903 159

Austria T: 01 68 66 250
F: 01 68 79 03
E-mail: serve.bioindat@eu.pharmacia.com

Belgium T: 3 272 14 69
F: 3 272 16 37
E-mail: serve.bioindnl@eu.pharmacia.com

Denmark T: 4814 1000
F: 4814 1006
E-mail: serve.bioinddk@eu.pharmacia.com

Finland T: 358 9 8520 7400
F: 358 9 8531 933
E-mail: serve.bioindfi@eu.pharmacia.com

France T: 0 1 69 35 67 00
F: 0 1 69 41 96 77
E-mail: serve.bioindfr@eu.pharmacia.com

Germany T: 0761 490 30
F: 0761 4903 405
E-mail: serve.bioindde@eu.pharmacia.com

Great Britain T: 01727 814 000
F: 01727 814 001
E-mail: serve.bioindgb@eu.pharmacia.com

Italy T: 02 273 221
F: 02 273 022 12
E-mail: serve.bioindit@eu.pharmacia.com

Netherlands T: 0165 580 400
F: 0165 580 401
E-mail: serve.bioindnl@eu.pharmacia.com

Norway T: 63 89 23 10
F: 63 89 23 15
E-mail: serve.bioindno@eu.pharmacia.com

Portugal T: 01 424 9200
F: 01 424 9299

Spain T: 93 589 07 01
F: 93 589 34 74
E-mail: serve.bioindes@eu.pharmacia.com

Sweden T: 08 623 85 00
F: 08 623 00 69
E-mail: serve.bioindse@eu.pharmacia.com

Switzerland T: 01 802 8150
F: 01 802 8151
E-mail: serve.bioindch@eu.pharmacia.com

Regional office Pharmacia Biotech Export (Vienna)
T: +43 1 982 3826 F: +43 1 985 8327
E-mail: rfeike@phexport-vie.co.at

CIS & NIS (Moscow) T: (international) 7 503 956 1137
(domestic) 7 095 956 1137
F: (international) 7 503 232 0250
(domestic) 7 095 232 0250

Czech Republic T: 02 205 11 392
F: 02 205 11 392

Hungary T: 01 1747 584
F: 01 1757 819

Israel T: 972 3 535 15 05 (ext 131)
[Gamidor Ltd]
F: 972 3 534 65 73

Poland T: 022 651 63 33
F 022 651 75 57

Ukraine T: 044 543 19 71
F: 044 418 10 76

Regional office Greece, Africa and Middle East

Athens T: 30 1 960 0687
F: 30 1 960 0693
E-mail: eliask@eexi.gr

internet: http://www.biotech.pharmacia.se

Asia Pacific

Main office Hong Kong
T: +852 2811 8693 F: +852 2811 5251

Asean Countries (Malaysia) T: 60 3 735 3972
F: 60 3 735 4672
E-mail: pbasean@po.jaring.my

Australia Tollfree: 1 800 252 265
T: 61 3 9887 3909
F: 61 3 9887 3912
E-mail:amradpb2@ozemail.com.au

Hong Kong T: 852 2811 8693
F: 852 2811 5251
E-mail: phhkmkt@hkstar.com

India T: 91 44 434 0747
F: 91 44 434 5537
E-mail: pharma@giasmd01.vsnl.net.in

Indonesia T: 62 21 384 8884
F: 62 21 384 9636
E-mail: hilabsci@indo.net.id

Malaysia T: 60 3 703 1888
F: 60 3 703 8047
E-mail: intersec@po.jaring.my

New Zealand Tollfree: 008 733 893
T: 64 9 638 7097
F: 64 9 638 7098
E-mail: amradpbnz@xtra.co.nz

People´s Republic of China T: 852 2811 8693
F: 825 2811 5270

(Beijing) T: 86 10 6256 4308
F: 86 10 6256 5603

(Guangzhou) T: 86 20 8760 1566
F: 86 20 8760 1566

(Shanghai) T: 86 21 6267 4621/4656
F: 86 21 6267 4611

Phillipines T: 63 2 634 6571
F: 63 2 635 4817
E-mail: secura@mnl.sequel.net

Republic of Korea T: 82 2 511 0801
F: 82 2 511 3711
E-mail: phdi@chollian.dacom.co.kr

Singapore T: 65 250 3330
F: 65 250 0003
E-mail: biolab@singnet.com.sg

Taiwan T: 886 2 831 6021
F: 886 2 831 5311

Thailand T: 662 615 2130
F: 662 271 4533
E-mail: becthai@ksc7.th.com

Vietnam T: 84 8 835 4652
F: 84 8 835 2997

Japan

Tokyo T: 03 3492 9499
F: 03 3492 9337

internet: http://www.biotech.pharmacia.se